

EFFECTS OF A RADIOSENSITIZER AND RADIATION ON THE CILIATED MUCOUS MEMBRANE

A comparative Physiological and Ultrastructural study

Maria Albertsson



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Title and subtitle Effects of a radiosensitizer and radiation on the ciliated mucous membrane. A comparative Physiological and Ultrastructural study.		
Abstract Fractionated irradiation (2 Gy/F, TD:2-20 Gy) in vivo on the ciliated epithelium of the rabbit's trachea, caused measurable physiological alterations ten consecutive days after completion of irradiation with an initial heightening of the ciliary activity after 2 and 4 Gy, followed by a dose-dependent decrease. On scanning electron microscopy-pictures, knobs were observed on the cilia with the number being related to the dose. The addition of misonidazole potentiated the effects described above, with an enhancement ratio(physiologically) of 1,2 and an enhancement ratio (morphologically) of 1,6. Furthermore, administration of misonidazole to the rabbits caused an increased vascularity in the subepithelial layer of the trachea, directly correlated to an oedema in the same region. Single Doses (2,2.5,5,10,15,20,25 and 30 Gy) were given to the trachea in vivo and daily investigations of the ciliary beating and morphologic examinations of the tissue were made for ten days. The ciliary activity showed a dose-dependent reduction of about 50% after 30 Gy. A development of damage, in relation to the dose, was observed in the cilia. The changes were blebs, swollen tips, bent and curved tips and broken cilia clustered together. During in vitro irradiation with 10 Gy Single Dose, an increase of the ciliary activity to about 25% of its original value was observed without any morphological changes, while in vivo irradiation and examinations during 10 days thereafter, showed three different phases, day 1-3:Stimulation phase, day 4-8:Damage-phase, day 9-10:Repair-phase. The ciliary epithelium offers an exceptional system for the study of early radio-biological effects, since mutual comparisons can be made between physiology and morphology.		
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To Lars

This work is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Scanning electron microscopy and recording of the physiological activity of tracheal ciliated cells treated by fractionated irradiation.

Albertsson M., Håkansson C. H. and von Mecklenburg C.

Scanning Electron Microscopy 1983:IV, 2019-2026.

II. The effects of 10 Gy single dose irradiation on the ciliated epithelium measured during and one-to-ten days following irradiation. A comparative physiological and morphological study.

Albertsson M., Baldetorp B., Håkansson C. H. and von Mecklenburg C.

Scanning Electron Microscopy 1984:II, 813-824.

III. Scanning electron microscopy and transmission electron microscopy of the ciliated cells of the trachea of the rabbit treated with misonidazole alone and in combination with ionizing radiation.

Albertsson M., Mercke C., Håkansson C. H. and von Mecklenburg C.

Radiotherapy and Oncology 1985:III, 47-60.

IV. Reaction of the vascular system in the trachea of the rabbit exposed to fractionated irradiation with and without the addition of misonidazole.

Albertsson M., Mercke C. and Håkansson C. H.

Accepted for publication in Radiotherapy and Oncology 1985.

V. Dose response studies of single dose ionizing radiation on the ciliated epithelium of the rabbit's trachea. A physiological and ultrastructural study.

Albertsson M.

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PAPERS I-V

INTRODUCTION

The most important progress in clinical radiotherapy originates from improvements in dose distribution mainly derived from the introduction of supervoltage radiation. This has made it possible to give a higher dose to tumour compared to adjacent tissues.

A number of biological parameters have been investigated in order to further improve the results. The efforts have focused on attempts at a selective augmentation of radiation effect on tumour tissue (or selective protection of normal tissues). Most widely studied of these factors have been dose/fraction relationship (39, 45, 80, 93, 138), dose/time relationship (8, 31, 35, 122), and the influence of the presence of hypoxic cells (56, 99, 126). Repair mechanisms in normal tissues and tumours (43, 44, 59, 132, 133, 135, 136) may play a role in the outcome of radiotherapy. Also cellkinetic parameters seem to have prognostic significance (115). However, it must be admitted that very little documented clinical progress has been evolved from these studies so far. Very few investigations have been pursued in the effect of radiation on physiological variables even if the term "radiophysiology" was mentioned as early as 1930 by Claude Regaud (102). Only a few tissue systems have so far lent themselves to such radiophysiological studies since they express their function in reasonably well defined and measurable ways. As examples of such systems, the registration of breathing rate and micturation rate in mice following irradiation of lung and bladder can be mentioned (50). Physiological changes after irradiation seem to reflect partly other aspects of radiation effect on tissues than are revealed with morphological methods or systems where cellular survival is the endpoint. In some of these it seems as if physiological methods can detect radiation effects earlier and with greater facility than is the case when morphological effects are studied (7, 116).

In the present investigation a similar system measuring the beat frequency of the rabbit's tracheal ciliated cells (physiological activity) has been chosen. This system has proven suitable for studies of early irradiation effects in vitro, where a stimulation of the ciliary beat frequency have been observed, varying in size with different radiation qualities and dose rate within seconds during irradiation, without any morphological changes (10, 11, 12, 13). In the actual work irradiation is



performed in vivo, and is a continuation of earlier in vitro studies. Physiological changes after irradiation has been correlated to structural changes as measured 1-10 days after completion of irradiation with light microscopy (LM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

THE AIM OF THE STUDY

In order to compare immediate radiobiological effects during irradiation in vitro with early effects 24-240 hours after completion of irradiation in vivo, and clarify dose/time relationship on the ciliated epithelium of the rabbit's trachea, single dose investigations have been carried out. The effects of fractionated irradiation have also been examined in order to imitate clinical circumstances. Furthermore, irradiation has been given together with misonidazole, a substance which has been thought to interact selectively with radiation in hypoxic tissue regions. This has been done in an attempt to gain additional knowledge of the basic mechanisms behind the action of this substance using a system for evaluation different from those used so far.

The investigation is presented in the following items.

I. Physiological and ultrastructural effects brought about by fractionated irradiation with a clinical conventional dose (2 Gy/F).

II. Effects of 10 Gy single dose during irradiation, directly after completion of irradiation and during the first ten days after completion of irradiation.

III. Physiological and ultrastructural effects of misonidazole, alone or in combination with irradiation.

IV. Effects on the vascular system in the trachea of misonidazole and irradiation in combination or separately.

V. Physiological and ultrastructural effects of Single Doses 2-30 Gy.

MATERIALS AND METHODS

This investigation comprises 400 rabbits weighing between 1.8-2.3 kg.

One-hundred rabbits were treated in groups of ten with fractionated irradiation (2 Gy/F) from a total dose of 2 Gy to 20 Gy (I, Fig. 1).

Another 100 were treated with the same fractionation schedule, however, misonidazole (100 mg) was injected prior to irradiation (III, Fig. 2).

Fifty rabbits were treated with misonidazole alone in a dose range from 100-1000 mg (III, Fig. 1).

Eighty rabbits were given radiation with doses ranging from 2-30 Gy (V, Fig. 1).

Forty rabbits were used for investigations of irradiation effects in vitro (II).

Thirty rabbits were used as control animals.

Irradiation technique: Irradiation was performed with a conventional X-ray therapy unit (SIEMENS). 160 kV X-ray, HVL:4 mm Al, SSD:50 cm.

Before irradiation, the animals had been rendered unconscious by an intraperitoneal injection of pentobarbital (40 mg/kg body - weight). The rabbits were laid on the back and the upper part of the trachea (20 mm) was irradiated. The caudal limit of the treated area was lead-shielded, and the direction of the beam was arranged at a cranial angle, in order to strike the caudal part along the cartilage arc. The spatial distribution between the irradiated part and the control part was 40 mm.

The absorbed dose in trachea and oesophagus was controlled by ion-chambers and thermoluminescent dosimeters. Beyond the irradiation field, the dose faded off sharply, and 15 mm beyond the caudal limit of the field, the absorbed dose was negligible.

Experimental procedure: The animals were irradiated in groups of ten. After completion of irradiation, one animal was taken out from the group on ten consecutive days.

The animal was sacrificed by a blow on the scull, in order to avoid pharmacological side-effects. The trachea was dissected out in its entire length (7 cm). Samples for SEM, TEM and LM were taken from the upper part of the trachea (irradiated area: T1) and lower part of the trachea (control area: T2). Control investigations were also performed in the same way on untreated animals. Thereafter the trachea was divided into two halves and

placed in a chamber with controlled temperature (37°C) and humidity (90 %). Through an opening in pars membranacea, the ciliary beat frequency could be recorded by a method developed and described by Håkansson and Toremalm 1965 (73). Briefly, heat filtered DC light from a cold light source illuminated the mucous membrane of the trachea via the incision and was reflected by the mucous ciliary border at the bottom of the trachea. The blinking light reflection which arose thereby due to the mucociliary activity was observed through a laboratory microscope. A photomultiplier attached to the lens of the microscope was connected to an ink plotter through a frequency filter. Registration of the mucociliary frequency was performed during 30 minutes from irradiated area and non-irradiated (control area) respectively. Such a registration was performed from at least two different places selected at random in each specimen. The mean value for the registration was shown as a quotient for each rabbit:

$$\frac{\text{beat frequency (irradiated area)}}{\text{beat frequency (control area)}}$$

Misonidazole was dissolved in sterile water and 100 mg was administered intraperitoneally, 15-30 minutes before irradiation.

Preparations for LM, SEM and TEM were made with routine methods earlier described (II).

The specimens were examined in a Cambridge Stereoscan MARK IIA, a Zeiss Nanolab Electron Microscope, a Zeiss EM 10 Electron Microscope and a Zeiss Light Microscope, on the institute of Zoology, University of Lund.

The tracheal epithelium consists of basal cells, intermediate cells and apical ciliated cells. Interspaced between these cells are the goblet cells. SEM-technique allows the surface to be evaluated with special attention to the appearance of the cilia, the microvilli covered goblet cells, mucus and detritus on the surface. TEM-investigations were used to evaluate the amount and appearance of the cellorganelles, while LM-sections gave an overview of the ciliated epithelium. The height of the ciliary cell layer, subepithelial lamina, subepithelial area and blood vessel area in the subepithelial layer was calculated from LM-sections (IV, Fig 8) and the ratio goblet cells/ciliated cells could also be calculated.

The Misonidazole Enhancement Ratio(physiology) = MiER(physiology) was calculated in the following way:

$$\frac{\text{mean value beat frequency : T1/T2 (misonidazole+irradiation)}}{\text{mean value beat frequency : T1/T2 (irradiation)}}$$

Such a ratio was calculated for each dose group (III, Fig6).

Misonidazole Enhancement Ratio(ultrastructure) = MiER(ultrastructure) originating from scoring different degrees of radiation damage on the SEM-pictures and was calculated for each dose group (III, Fig 13) from:

$$\frac{\text{score mean value (misonidazole+irradiation)}}{\text{score mean value (irradiation)}}$$

Temperature measurements. The temperature was measured with a thermistor beneath the pars membranacea, where radiation had been administred and this temperature was then compared to that of the corresponding non-irradiated area at the distal part of the trachea.

Determination of bodyweight. The weight of the animal was checked before the treatment and daily after the treatment had been given.

Statistical analyses of the results were made with the help from statistical expertise from the University of Lund.

RESULTS

Scanning electron microscopy and recording of the physiological activity of tracheal ciliated cells treated by fractionated irradiation(I).

Fractionated irradiation (2 Gy/F) was given from a total dose of 2 Gy up to 20 Gy. Investigations were performed 1-10 days after completion of irradiation. In the low dose range (2 Gy, 4 Gy), the beat frequency of the cilia was increased within the irradiated area ($T1/T2 > 1$). Additional irradiation reduced the ciliary activity ($T1/T2 < 1$). Variance analysis of the whole material showed a significant dose-dependent reduction with increasing dose, however no significant time effect. LM showed dose-dependent oedema of the ciliary epithelium, noticable already the first day after 2 Gy and most pronounced after 20 Gy (I, Fig. 3, 4). The number of goblet cells was also heightened with increasing dose (I, Fig. 10, Table II). SEM showed blebs on the cilia, in relation to the total dose given (I, Table I, Fig. 6-9). In the low dose range, the number of mitochondria in the apical part of the ciliated cells was augmented.

The dose-dependent decrease of the ciliary activity and the dose-related increase of blebs on the cilia observed on SEM-pictures is evident. However, a correlation between the entities could not be revealed and therefore further experiments were performed.

The effects of 10 Gy single dose irradiation on the ciliated epithelium measured during and one-to-ten days following irradiation. A comparative physiological and morphological study (II).

Recording of the beat frequency during irradiation with roentgen (50 kV) and ^{60}Co were made in order to compare effects of low X-ray irradiation and high energetic irradiation. The low-voltage photons caused an increased beat frequency within the irradiated area of about 25% after an accumulated dose of 1.5 Gy. After a dose of 3 Gy it returned to a level just above the value before irradiation (II, Fig. 1). The ^{60}Co -irradiation caused a corresponding increase of about 22% after an absorbed dose of 1.3 Gy. The increment lasted longer and returned more slowly than after roentgen irradiation (II, Fig. 2). No structural changes could be seen in these investigations of immediate effects during irradiation.

Investigations of late effects during ten consecutive days after 10 Gy single dose were considered to show three different phases.

1: Day 1-3 (II, Fig. 3) which was called a stimulation phase, during which the beat frequency was slightly above the normal value (the beat frequency in the non-irradiated part of the trachea).

2: Day 4-8 was regarded as a damage phase with disorganisation, adhesion of the cilia and accumulation of mucinous granula (II, Fig. 7, 8). The transport mechanism was reduced as well as the ciliary activity in those areas where it could be measured (II, Fig. 3).

3: During day 9 and 10 there seemed to be some kind of recovery, when the beat frequency and ultrastructure normalized.

Scanning electron microscopy and transmission electron microscopy of the ciliated cells of the trachea of the rabbit treated with misonidazole alone and in combination with ionizing radiation (III).

Irradiation of the ciliated epithelium of the rabbit's trachea caused dose-dependent physiological and ultrastructural effects in relation to the radiation dose as shown in paper I. The addition of misonidazole caused a potentiation of these effects. The effects of misonidazole + irradiation as compared to irradiation alone, showed for all irradiation doses a heightening of the beat frequency (III, Fig. 6). This could be expressed as an enhancement ratio (physiologically, see Material and Methods), with a mean value of 1.2 (all dose groups). Changes observable with SEM were scored and also here a potentiation could be seen when misonidazole was added before the irradiation (III, Fig. 3). The enhancement ratio (ultrastructurally, see Material and Methods), was calculated to 1.6 as a mean value for all doses.

TEM-investigations of the group treated with misonidazole + irradiation seemed to show an increased metabolic activity as registered by heightening of the number of mitochondria, a rich amount of endoplasmic reticulum and free ribosomes and multilobulated nuclei with an increased number of nuclear pores and a greater number of goblet cells than normal (III, Fig. 15 a, b, c). Also a hyperplasia of the epithelium was seen (III, Fig. 7, Table III). Misonidazole alone caused no changes in the beat frequency. Morphological changes could not be observed on SEM, but LM and TEM showed a hypoplasia (III, Fig. 5, Table III).

The potentiation of irradiation effects observed in this study when misonidazole was added before the irradiation is incontrovertible. However misonidazole itself has no stimulatory effects on the ciliary beat frequency or the metabolism within the cell as registered by lack of changes on TEM-pictures.

Reaction of the vascular system in the trachea of the rabbit exposed to fractionated irradiation with and without the addition of misonidazole (IV).

Fractionated irradiation (2 Gy/F, Total Dose 2-20 Gy) caused an oedema of the ciliary cell layer and subepithelial lamina observed in LM-investigations (IV, Table 1, 2). The vascular area in the subepithelial layer was calculated and was of the same magnitude as in the control material (IV, Fig. 10). In the ciliary cell layer, subepithelial area and subepithelial lamina an oedema was observed, and the magnitude could be calculated from LM-pictures. Correction for the oedema showed a slight increase in the vascularity in the irradiated group as compared to the control group (IV, Fig. 10). However, no correlation between the oedema and the vascular area was found in the irradiation group. Treatment with misonidazole alone or in combination with radiation caused a marked increase in the vascularity in the subepithelial layer of the trachea (IV, Fig. 6, 7, 10). In the misonidazole treated groups an oedema was also observed in the ciliary cell layers and subepithelial lamina, directly correlated to the vascularity. The increased vascularity in the groups treated with misonidazole was significant with or without correction for oedema. To our knowledge this finding has not been described before.

Dose response studies of single dose ionizing radiation on the ciliated epithelium of the rabbit's trachea (V).

A physiological and ultrastructural study.

Single doses of 2, 2.5, 5, 10, 15, 20, 25, 30 Gy were given and daily investigations were made for 10 days (V, Fig. 1). Low doses (2, 2.5 Gy), during the first days after irradiation, caused an augmentation of ciliary beat frequency within irradiated area (V), which during the following days

returned to the normal value. Ultrastructurally, blebs could be observed on the cilia after 1-10 days (V, Fig. 4a, b, Fig. 9). Five and ten Gy of irradiation also seemed to have a stimulating effect on the ciliary activity (V, Table 1, 2). In this dose range however, the structural effects were more pronounced with swollen ciliary tips, bent tips, some cilia broken and adhering to each other (V, Fig. 5 a, b, Fig. 6, Fig. 7 a, b, c, Fig. 8 a, b, Fig. 9, Fig. 10). Larger single doses (15, 20, 25 and 30 Gy) caused reduced ciliary activity and after 30 Gy a reduction of about 50% (V, Fig. 2, Table 1, 2). After these doses the ciliary activity was irregular and ultrastructurally most cilia were broken and adhered to each other (V, Fig. 9, Fig. 10). The radiation effects also included the goblet cells, with a greater amount of these cells after low doses (V, Fig. 12 a, b, Fig 15). Higher doses ≥ 10 Gy caused emptying of the goblet cells which made an estimation of their number on SEM-pictures difficult to evaluate (V, Fig. 13), and mucinous granula on the surface (V, Fig. 14 a, b, Fig 15).

In the above described single dose investigation, dose- dependent reduction of the ciliary activity seemed to be related to a development of ultrastructural damage on the cilia with blebs, swollen tips, bent, broken and adhering tips. However in the higher dose range ≥ 15 Gy when most of the ciliary carpet was put out of action, no reparative processes were observed during the ten day observation period, which indicates that further experiments with prolonged observation period must be performed for investigations of repair and regeneration.

DISCUSSION

In earlier radiobiological research work concerning the influence of radiation on normal tissues a vast number of methods and systems have been used, mainly concentrating on effects after weeks, months and years studied on a macroanatomical level. The epidermis has been one of the most widely studied normal tissues, (29, 30, 32, 33, 48, 49), which probably depends on the fact that this tissue is easily accessible for observation, and damage to it can easily be scored on an arbitrary scale. Another system to visualize early radiobiological effects has been used in this study where comparative studies of physiological and ultrastructural radiation effects can be performed on the ciliated epithelium of the rabbit's trachea. Alterations in the physiological activity can be evoked within seconds during irradiation in vitro (12). It seems also suitable for measurements of early physiological radiation effects, i.e. 1-10 days after completion of irradiation in vivo where a direct comparison can be made with ultrastructural effects observed with SEM and TEM. A necessary presumption for this investigation, is knowledge of the normal physiology and ultrastructure. These parameters have been extensively studied in earlier research work (73, 74, 75, 76, 84, 85, 103, 109, 110, 111, 112, 129, 130). Radiobiological research with this system has earlier been done on a dissected rabbit's trachea in vitro (10, 11, 12, 13). The purpose of the present investigation was to some extent to imitate the clinical circumstances and therefore irradiation was performed in vivo with fractionated irradiation alone or in combination with a radiosensitizer (misonidazole). In order to get a complete grasp of the radiobiological effects in the actual tissue, single dose investigations were also carried out.

Treatment with fractionated irradiation caused an increase in the beat frequency the first days after completion of irradiation with 2 and 4 Gy to about 10% (I). This was correlated to an increased number of mitochondria apically in the ciliated cells (I, III). The heightening of the beat frequency may depend on effects on the energy-production system within the cell, with an increased amount of ATP released from the mitochondria. ATP is the energy source of the cilia and the supply of ATP governs the ciliary beat frequency (18, 19, 70). Experiments have been performed in order to investigate physiological effects during and after irradiation in vitro.

During irradiation an increased frequency of about 25% was seen after 10 Gy (50 kV X-rays; II) and 22% after 10 Gy (^{60}Co - irradiation; II). The acceleration of beating within seconds during irradiation could be ascribed to a mechanism within the cilium itself (12), perhaps an increased available amount of dynein ATP-ase, or by changes in the membrane permeability of the cilia, allowing some ionic fluxes which could alter the rate of ciliary beating. Immediately after irradiation the frequency decreased (53, 89; II, Fig 14). It therefore seems plausible that the increased ciliary activity during irradiation with photons of different energies is caused by mechanisms within the cilium since no morphological changes can be observed while the increased ciliary activity measured 24 hours after completion of irradiation seems to be related to the cell metabolism, with a heightened number of mitochondria apically in the ciliated cells.

With increasing total dose, the beat frequency decreased ($T_1/T_2 < 1$), and in the whole material a significant dose-effect was found, but no significant time effect. All the quotients (ciliary beat frequency (irradiated area)/ ciliary beat frequency (control area)) in this series (I) were further analyzed in two separate parts in order to see if there were any different effects concerning dose and time in the lower dose range (2-10 Gy) or higher dose range (12-20 Gy). For 2-10 Gy, neither dose-effect nor time effect was observed. In the series 12-20 Gy, however, a significant dose-effect with decreasing beat-frequency in relation to increasing dose was found and a significant time-effect with increasing beat frequency in relation to increasing time. The inverted relationship between beat-frequency and dose may be related to several mechanisms where the oedema probably plays a role (I), in combination with blebs on the cilia. Also a consumption of the available ATP and a lack of newly synthesized ATP can contribute to these results. The time-effect may be attributed to compensatory proliferation. Such a mechanism is known to start within 2 days in the intestine (136, 137) and in the skin within 1-2 weeks (30, 31, 33). It can also be observed in the clinic where desquamation and reparative proliferation of e.g. buccal mucosa may occur during a fractionated irradiation schedule (35). If the increased beat frequency towards the end of the observation period in the high dose range is caused by repopulation, it starts within 3 weeks after start of irradiation. Another mechanism that may cause the same effects would be a repair mechanism and since 1959 when

Elkind and Sutton first described the repair of sublethal radiation damage (44), several other repair processes have been identified varying in time scale. Fast repair occurring within seconds is observed in bacteria. Elkind-repair has been described for bacteria and mammalian cells in vitro, and for normal and malignant tissues in vivo (9, 43, 44, 95, 135, 136), and takes place within minutes to hours after irradiation. Other repair mechanisms are potentially lethal damage (PLD-repair), taking place within minutes to hours and observed by delaying postirradiation division usually by altering the nutrient status (60). A slow repair process has been observed in the lung (46, 50, 96), with a time scale of hours to days. To evaluate whether the repair of radiation-induced damage takes place in the tracheal tissue from the known definitions of repair seems to be difficult. It is plausible that some form of repair, with the definitions described above, exists.

The increment of the beat frequency quotients towards the end of the observation period ten days after completion of fractionated irradiation 12-20 Gy, is believed to be caused by repopulation rather than repair for several reasons, e.g. the time scale in which the event takes place (about 2-3 weeks) and the similarities to the reactions observed in the skin during fractionated irradiation (31). SEM of the cilia during fractionated irradiation showed blebs or knobs on the cilia, which were small swellings situated at the sides or the tips of the cilium (I, Fig. 6-9), increasing in number with increasing dose (I, Table 1). Blebs are observed in the normal epithelium to a lesser extent (27), and may be weak regions on the cilia that are more easily affected by radiation. Membrane damage from ionizing radiation is earlier described both on an ultrastructural level (17, 47, 61, 63, 128, 134), and by measuring leakage of proteins from blood vessels after irradiation (88, 105, 116, 117). Membrane-damage is probably also the reason for the oedema in the ciliated epithelium and the subepithelial layer, observed after fractionated irradiation (I, Fig. 4). Treatment with single doses from 2-30 Gy (V, Fig. 1), made it possible to find a pattern in the development of ultrastructural changes where blebs seem to be an early phenomenon of damage, observable already after such a low dose as 2 Gy. After doses > 15 Gy, this can no longer be observed to a greater extent than was observed in the control animals. However, after 30 Gy almost all cilia are short, stubby, broken and adhere to each other, which may indicate that

broken cilia and adhesion are the most pronounced damage effect (V, Fig. 7, 8, 10). After the single doses 2-30 Gy, a dose-effect could be observed with decreasing beat frequency and increasing structural damage with increasing dose. No time effect could be seen in the single-dose investigations and the postulated repair phase, day 9-10 after 10 Gy single dose, is probably a kind of recovery, where the cilia regain their normal appearance after a period of shrinkage. The lower score on the SEM-pictures may depend on a smaller amount of mucus on the surface during those days (V, Fig 15). A stimulation of goblet cells and emptying of mucinous granula seen after irradiation, has been earlier described as existing in the intestine (52). In the actual experiment the same stimulation was found (I, TableII, V, Fig 15). The mechanism behind it is not clear, nor is it known whether goblet cells are produced from undifferentiated basal cells or differentiate from intermediate cells (107). The great amount of mucus probably plays a major role in determining the ciliary activity, perhaps causing changes with a higher viscosity (108). On the whole, single doses (2-30 Gy) cause reproducible structural and physiological effects on the tissue. One single or a few massive doses were the conventional methods used in the early days of radiotherapy, which often caused excessive normal tissue damage. However, already in 1922 and 1929 Regaud (100, 101) showed that a multifraction regimen caused more damage to rapidly proliferating tissue (gonadal cells) whilst sparing the more slowly proliferating surrounding tissue (skin). It is now generally accepted that a multifraction regimen causes more damage to the tumour than to the surrounding tissue, because reoxygenation of hypoxic cells in the tumour occurs during treatment, while repair and repopulation in the normal tissue during treatment, spare this tissue. Hypoxic cells in tumours are a major impediment in local tumour control by radiotherapy (99, 126). The three main ways of dealing with hypoxic cells are (51):

- 1: Radiotherapy in hyperbaric oxygen (23, 24).
- 2: Neutrontherapy and heavy-ion radiotherapy (14, 15, 83, 120).
- 3: Radiosensitizers of hypoxic cells (1, 2, 3, 4, 6).

Hyperbaric oxygen and neutrontherapy have shown contradictory results. However it has been shown that hypoxic cells can be a problem for patients with certain kinds of malignant tumours (22, 24, 57, 58, 69, 131). Therefore it might be worthwhile to develop other ways of dealing with hypoxic cells.

Electron-affinic drugs with "oxygen mimic" qualities (metronidazole, misonidazole) have been studied together with low LET radiation for the treatment of human tumours, so far with disappointing results (38, 97). However new compounds, some electron-affinic with a chemical structure similar to misonidazole are under development with a higher therapeutic ratio because of lower neurotoxicity (5, 26, 36, 40, 64, 79, 98).

In the actual experiment, the combination of misonidazole and radiation caused a potentiation of the effects on the physiological activity as represented by increased beat frequency (III, Fig. 6). These findings made it possible to define a new epitome: $MiER(Physiology) = \text{Misonidazole Enhancement Ratio(Physiology)}$. In this case $MiER(physiology)$ was found to be 1.2 (mean value of $MiER$ for all doses (III, Fig 6)).

A similar relation was based on the ultrastructural investigations, originating from the scoring of the SEM-pictures (III, Fig. 13), which was called $MiER$ (ultrastructure), and found to be 1.6.

Signs of increased metabolic activity were more prominent in the group treated with misonidazole + irradiation as compared to the irradiation group. These signs were an increased amount of mitochondria in the apical ciliary cell layer (III, Table II, Fig. 14), increased smooth and rough endoplasmic reticulum (III, Fig. 15c), multilobulated nuclei and an increased number of nuclear pores (III, Fig. 15b). A hyperplasia of the ciliary cell layer was also seen (III, Table III, Fig. 7).

LM investigations showed an increased vascularity (IV, Fig. 10) in the subepithelial layer which was correlated to an oedema (IV, Table 1, 2) and observed throughout the series without any dose-dependency. The number of blood vessels stayed unchanged. Therefore the increased vascularity may either depend on vascular stasis, which would lead to hypoxia or an increased blood flow (hyperemia) with an improved oxygen delivery to the tissues. The potentiation of radiation effects measurable both physiologically and ultrastructurally speaks in favour of the latter alternative. Improved oxygen delivery to tumour tissue and subcutaneous adipose tissue in tumour bearing mice, after administration of misonidazole, has earlier been observed (28) and metronidazole-treatment of mice with

C3HBA mammary adenocarcinoma caused improved oxygenation of the tumour (65). It has been shown that the drug can alter cellular oxygen consumption in vitro (42, 81), and inhibition of cellular oxygen consumption through decreased overall metabolic rate can alter cellular oxygen consumption and lead to an improvement of radiation response (16). It has also been shown that administration of metronidazole to mice produced a drop in the rectal temperature and heart rate, which was interpreted as a result either from a overall decreased metabolic rate or a peripheral vasodilatation (54). If the potentiated irradiation effects observed on the ciliated epithelium of the rabbit's trachea were caused by an overall decreased metabolic rate, an increase of the vascular area and signs of an increased metabolism should not have been observed. This finding supports our hypothesis that $MiER(\text{physiology}) = 1.2$ and $MiER(\text{ultrastructure}) = 1.6$ may be caused by a hyperemia with an improved oxygen delivery to the tissue.

Misonidazole has shown a sensitization of the normal tissue reaction after irradiation in several investigations e.g. in skin (20, 68, 119, 123, 140), testis (125), mouse tail (66), tibial cartilage (55), spinal cord (141) and oesophagus (72). However in the intestine, both metronidazole and misonidazole have shown a protective effect (52, 121)

Treatment with misonidazole alone also caused an increased vascularity (IV, Fig. 10) which were correlated to an oedema in the subepithelial lamina. TEM-investigations of the trachea of rabbits treated with misonidazole caused a hypoplasia of the ciliary cell layer with lack of intermediate cells (III, Table III, Fig. 5). This may be a cell-killing effect which is observed after misonidazole-treatment both for hypoxic cells (62, 87, 92, 118, 124) and oxic cells (21, 90). Another explanation could be a general lengthening of the cell cycle time. This may be the case in the actual experiments and is supported by reports that such mechanisms have been found during both hypoxic (94), and oxic conditions (37, 82).

The mechanism of misonidazole causing cytotoxicity to well-oxygenated cells is not known. The cytotoxic effects of the drug are believed to be a consequence of reductive metabolism and therefore have hypoxia as prerequisite, but a hypothesis is that toxic metabolites can spread over from hypoxic to oxic regions where they exert cytotoxic effects. The

toxicity most well-known so far, is on the nervous system, giving convulsions and EEG-changes in the human together with neuropathy, i.e. axonal degeneration and demyelination of the peripheral nervous system (25, 113, 127).

All the animals in this study treated with irradiation, have also received pentobarbital (40 mg/kg bodyweight). It is known that the drug can alter the physiology in such a way that changes in radiation response may occur. Radioprotection has been observed in tumours (34, 86, 91, 106, 114) and in normal tissue (7, 41, 78, 104). Also both in normal and tumour tissue radiosensitization or no effect from anaesthesia has been seen (29, 34, 67, 77). Different mechanisms have been proposed as reasons for the changed radiation response. The protection has often been ascribed to physiological alterations leading to an oxygen deficit (77, 106, 142). Investigations with multicellular spheroids have shown hypoxic radiosensitization and oxic radioprotection, which was suggested to depend on a suppression of respiration in the oxic cells and a consequent improvement in oxygen supply to previously hypoxic cells (139).

An eventual effect of the anaesthesia in the interpretation of the results described here, would possibly be expected. Therefore separate analyses have been performed both of 30 control animals and of all control preparations in the lower part of the trachea (T2) in animals treated with fractionated irradiation (I, Fig. 1). All of these investigations showed no influence of the beat frequency, no oedema, no increased vascularity and normal ultrastructure. In these control investigations, no influence of the anesthesia could be seen 24-240 hours after completion of irradiation (Lars Henningsohn, pers.com.).

CONCLUDING REMARKS

The experiments performed in this radiobiological study have shown the following results:

1. Fractionated irradiation to the rabbit's trachea *in vivo* (2 Gy/F, 2-20 Gy) and investigations 1-10 days after completion of irradiation exerts an initial stimulation followed by a dose dependent reduction in the mucociliary activity (physiological effect) and evokes structural changes manifested as blebs or knobs on the cilia together with an oedema of the epithelium (morphological effect).

2. The addition of misonidazole just before the fractionated irradiation (2 Gy/F, 2-20 Gy) speeds up the beat frequency of the ciliary movement which allows the formulation of an enhancement ratio factor MiER(physiology). The value came out at 1.2. Misonidazole alone has no effect on the beat frequency.

A greater degree of damage to the tissue after addition of the sensitizer is revealed and a score of the average reaction based on the SEM pictures gives an enhancement ratio MiER(ultrastructure) of 1.6. SEM-investigations after treatment with misonidazole alone show no effects.

3. One of the pharmacological actions of misonidazole in the subepithelial layer of the rabbit's trachea appears to be a vasodilatation, the discovery of which seems to be a newly acquired comprehension.

4. An acceleration of the ciliary beat frequency within seconds is evoked during in vitro-irradiation with roentgen and ^{60}Co -quantities (10 Gy single dose) without any change in the morphology as seen on the ultrastructural level.

After irradiation in vivo (10 Gy roentgen) and registration of effects during ten consecutive days after completion of irradiation it is assumed that three phases could be discovered: day 1-3 stimulation phase with the ciliary beat frequency slightly above the normal value, day 4-8 damage phase with disorganisation of the cilia and accumulation of mucinous granula. The ciliary activity was reduced in those areas where it could be measured. Day 9-10 repair phase with a normalization of the beat frequency and ultrastructure.

5. Irradiation with single doses 2-30 Gy causes a gradual decay of the ciliary effectiveness related to an increased damage of the tissue. However, even after 30 Gy the mucous membrane is not totally disintegrated and some kind of activity can be observed in the cilia.

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SCANNING ELECTRON MICROSCOPY AND RECORDING OF THE PHYSIOLOGICAL ACTIVITY
OF TRACHEAL CILIATED CELLS TREATED BY FRACTIONATED IRRADIATION

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Abstract

The ciliated epithelium of the rabbit's trachea was irradiated with daily fractions of 2 Gy to an accumulated dose of 20 Gy. The beat frequency of the cilia was recorded and specimens were taken for SEM-, TEM- and LM-investigations. Examination was made 1-10 days after each fractionation schedule. An increased ciliary beat frequency was recorded at 2 Gy and 4 Gy. With increasing dose, there was an inverted relationship to the frequency.

Light-microscopy showed edema and an increased amount of goblet cells in relation to the increasing dose. With SEM an increased number of ciliary blebs could be seen. These could be classified according to size and number, and showed a positive correlation to the dose. TEM-investigations showed signs of increased intracellular activity at higher doses in the form of multilobulated nuclei and an increasing number of nuclear pores. At lower doses, an increased amount of mitochondria appeared in the apical part of the cell.

It is at present difficult to evaluate any correlation between the physiological activity and the morphology. More biological data are needed to explain the early irradiation effects.

Introduction

In radiobiology, different schedules of fractionation have been tested in the hope of improving radiotherapy. They have been based on clinical experience and increasing knowledge about the various events in the cell cycle. Repopulation and reoxygenation are thought to occur when irradiation of malignant and normal cells is spread out over a time period. Empirically a multifraction regimen is less injurious to the normal tissue and is more effective on the tumor cells. The normal fractionation pattern is 2 Gy, (1 Gy = 100 rad = the unit of absorbed dose corresponding to an energy absorption of 100 ergs/g) given five days a week.

Many experiments have been performed during the years concerning dose-time relationship. Strandqvist (1944) worked out a diagram where, for example, healing from tumor growth, necrosis and erythema were parameters for evaluating the irradiation results regarding dose and time. Ellis (1969) expressed the late results of radiation in dose, time and number of treatments. Tolerance and subtolerance levels have been studied on this basis by Kirk et al. (1971) and Turesson (1978), but all of their investigations are based on biological events at a late time (days, weeks), and the effect recorded on the macroanatomical level. These authors furthermore calculated with an interrupted fractionation (5 d/w, 6 d/w).

This paper deals with the effect of fractionation on the physiological activity and the morphological appearance of a tissue (rabbit's trachea) studied on the ultrastructural level in an attempt to find an eventual correlation between these two parameters in relation to dose, number of fractionations and time after irradiation.

Materials and Methods

This study investigated 100 rabbits, which were treated in groups of ten with fractionated irradiation of 2 Gy/F. The series extends from 2 Gy to 20 Gy. The fractionation schedule is presented in Fig. 1. One rabbit per day was taken out of the group 1-10 days after completed irradiation. It was sacrificed and the beat frequency of the cilia was recorded. Specimens were taken for SEM- and TEM-examination. Irradiation was performed by 160 kV X-ray: HVL: 4mm Al, SSD: 50 cm.

Key Words: cilia, tracheal epithelium, irradiation, scanning electron microscopy, transmission electron microscopy, physiological activity.

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Gy	Day after irradi.	1	2	3	4	5	6	7	8	9	10
2		.	.	.	.	.	.	.	.	.	.
2+2		.	.	.	.	.	.	.	.	.	.
2+2+2		.	.	.	.	.	.	.	.	.	.
2+2+2+2		.	.	.	.	.	.	.	.	.	.
2+2+2+2+2		.	.	.	.	.	.	.	.	.	.
2+2+2+2+2+2		.	.	.	.	.	.	.	.	.	.
2+2+2+2+2+2+2		.	.	.	.	.	.	.	.	.	.
2+2+2+2+2+2+2+2		.	.	.	.	.	.	.	.	.	.
2+2+2+2+2+2+2+2+2		.	.	.	.	.	.	.	.	.	.
2+2+2+2+2+2+2+2+2+2		.	.	.	.	.	.	.	.	.	.

Figure 1. Fractionation schedule. Each day 2 Gy was given and examination made the day 1-10 after irradiation.

Irradiation ciliated area: 2 cm of the cranial part of trachea. Each rabbit was rendered unconscious for about 15 minutes with 40 mg/kg body-weight Pentobarbital given intraperitoneally before each irradiation. The animal was laid on its back with the extremities stretched out. The absorbed dose in the trachea was controlled by an ion-chamber. For ten consecutive days, one rabbit was sacrificed by a blow on the skull, and the trachea was immediately dissected. Samples for SEM and TEM were immediately taken, both from irradiated and non-irradiated areas for comparative studies. The trachea was then placed in an experimental chamber with controlled humidity (90%) and temperature (37°C). The ciliary frequency was recorded within the irradiated area and also in an area outside the irradiated field,

where the absorbed dose was negligible.

Preparation for SEM

The specimens were transferred into 2.5% glutaraldehyde in 0.15 M cacodylate-buffer (pH = 7.3), for 12 hours. They were then transferred into the same buffer and later osmium fixed in 1% osmium tetroxide in 0.15 M cacodylate-buffer for two hours.

Dehydration took place with graded series of ethanol and then transferred to Freon TF 618. Later the specimens were dried according to the critical point method in a Balzers 000 Critical Point Dryer. They were finally sputter-coated with gold plus palladium in a Polaron SEM Coating unit E5000. Then they were examined in a Cambridge Stereoscan MARK IIA, or a Zeiss Nano-lab Electron Microscope. The microscopes were operated at 20 kV.

Preparation for LM

Sections for LM were fixed and embedded as for TEM sectioning. 1 µm thick sections were cut with an LKB-Ultratome and stained in methylene-blue and examined in a Zeiss light microscope. The mucociliary activity was recorded directly after dissection, in the experimental chamber. The method used was the indirect reflection method developed and described by Håkansson and Toremalm (1965). The ciliary beat frequency within the irradiated area was recorded and compared with the frequency of the cilia with non-irradiated areas (bifurcation of the trachea) in the same animal.

Statistical analysis

The statistical analysis of the material was performed by analysis of variance and related methods using the well-known program package BMPD.

Results

The effect of irradiation on the beat frequency

In the non-treated trachea, the beat frequency of the ciliary activity was the same as that measured at the bifurcation. It varied at 37°C between 800-1026 beats/min. with a mean of 860 S.D.50. The first day after irradiation with 2 Gy, the irradiated area showed an increased frequency in relation to the non-irradiated part (12%). With increasing dose, it was replaced by a gradual reduction. As an example of this Fig. 2 is given from the first day after irradiation where the regression line is:

$$y = -0.012 X + 1.039$$

The statistical analysis of the whole material showed a significant effect of the given dose on the beat frequency. This diminished linearly with a significance of $p < 0.0001$. The ciliary frequency of the non-irradiated part of the trachea was unchanged.

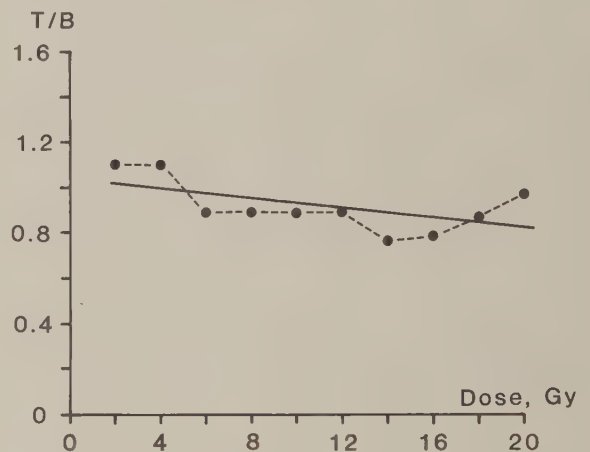


Figure 2. The ciliary beat frequency within the irradiated part of the trachea (T) in relation to the beat frequency of the non-irradiated bronchial part (B). One point is the experimental data from one rabbit and the ten results are obtained from the first day after finished irradiation. The solid line constitutes the regression line.

Edema as a result of irradiation

A difference was found between the irradiated trachea and the bronchus in regard to the thickness of the epithelium. With increasing dose the irradiated epithelium became thicker during the first days. This phenomenon was noticed already the first day after 2 Gy and became more pronounced in relation to the increasing dose. Twenty-four to forty-eight hours after 20 Gy, the relation between the thickness of the irradiated and the non-irradiated epithelia, was about 2/1. This gradually decreased as time passed

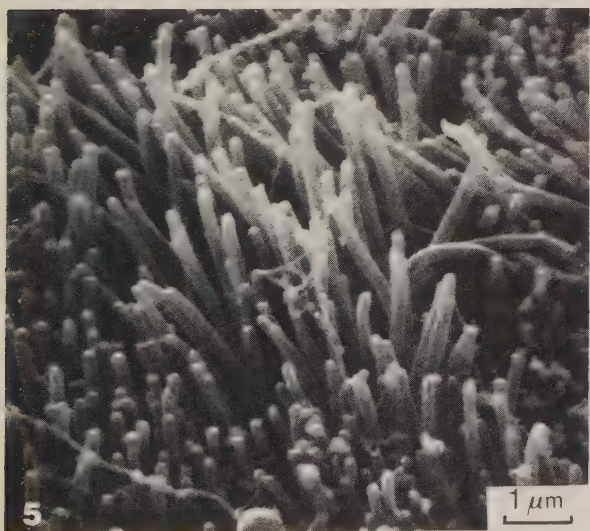
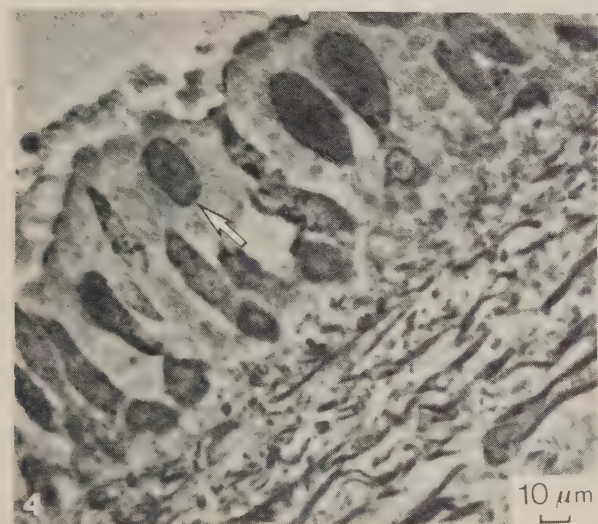
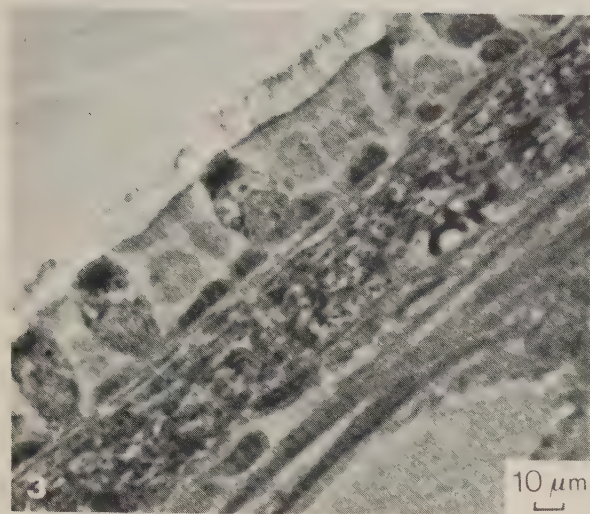


Figure 5. Cilia from non-irradiated rabbit trachea.

Figure 3. Light microscopy of the non-irradiated ciliary epithelium at the bifurcation area. Figure 4. Light microscopy of the ciliary epithelium from the animal shown in Fig. 3, irradiated with 18 Gy and examined 1 day after irradiation. Note the amount of goblet cells (arrow) and the edema.

but could clearly be detected at day 10 after 20 Gy. (Figs. 3 and 4).

Knobs on the cilia

Since there are discussion about the etiology of knobs on the cilia, if they are artefacts or a result of assault on the ciliary membrane, special attention was made to discover any difference in the presence of these extrusions on the cilia in irradiated and in non-irradiated areas. The blebs were usually small swellings situated at the top of the cilia, but they were also observed on the side of a cilium. Blebs were found both in irradiated and in non-irradiated areas. It was not difficult to observe that the blebs occurred more frequently in those areas irradiated with 20 Gy than with 2 Gy, but it was difficult to make more than a rough quantitative calculation of their number.

The estimation was best made on the SEM-picture. It was assumed that 5-25 blebs could be detected on a 12,000 x picture, on a determined area. (Fig. 5). This was scored as 1+. It was found that all controls could be included in this group.

When judging irradiated areas, it was found that swelling of the tips occurred more often at a higher dose, as well as clustering of the blebs (Figs. 6 and 7). If a scoring from 1+ to 3+ was made, the estimation of the frequency of blebs was as follows (Table 1).

Table 1

Gy	Irradiated	Non-irradiated
2 - 6	+(+)	+
8 - 12	+ +(+)	+
14 - 20	+ + +	+

Table 1. Estimations of the number of blebs at different doses on the cilia of the irradiated and the non-irradiated control areas.

+ = 5-25 blebs

++ = 26-75 blebs

+++ = > 75 blebs

as measured on a determined area on a 12,000 x picture.

The first days after irradiation, the blebs at highest doses seemed mostly to be confined to the tips of the cilia. (Fig. 8-9).

It was not possible to classify the different stages as described by Dalen (1982) in the development of the knobs in relation to dose. All stages were represented at all doses.

Goblet cell and irradiation

Goblet cells responded to irradiation and showed an increasing physiological activity, the larger the dose. In the normal trachea, goblet cells are interspaced between the tracheal cells in the proportion of ten ciliated cells to one goblet cell. Already the first day after 2 Gy, the proportion of goblet cells began to increase. This could be measured the following days until the tenth day. With increasing dose, the number of goblet cells appeared to increase, and this was seen throughout the ten days (Fig. 10). For the entire schedule (Fig. 1), the density of goblet cells can be presented as follows (Table 2 and Fig. 3).

At these doses, no sign of exhaustion could be detected, and the emptying of the cells was apocrine.

Physiological activity interpreted by TEM

Mitochondria were scattered throughout the whole ciliated cell, but showed a tendency to be accumulated at the apex. Irradiation seemed to increase the number, preferably beneath the ciliated cell membrane. This could be seen already the first day after 2 Gy. It was difficult, or even impossible, to exactly evaluate their number but this could be regarded as a clear finding, especially in the lower dose range. (Fig. 11).

Signs of increased metabolic activity in relation to the dose could be detected. Figure 12 shows multilobulated nuclei and an increased number of nuclear pores. The presence of an increased ribosomal activity has been difficult to relate to the irradiation. Inside the nuclei, zones with less density were seen (Fig. 11b, 14). Those zones were not detectable in the low dose range. They could be discovered at 6 Gy ten days after irradiation, but not at 6 Gy the first day after irradiation. At higher doses, they were more frequently represented in the dispersed chromatin. Single ones were also detected in the non-irradiated region. Heterophagic vacuoles were seen (Fig. 11a, 13) in an increased number in those cells which had received a high dose.

Discussion

The increased activity of the ciliary beating the first two days after 2 Gy and 4 Gy signifies an increase of the physiological activity caused by the irradiation. Stimulation as an effect of radiation was proposed as early as 1898 (Maldiney and Thouvenin) and 1903 (Bohn), but the question of "stimulation" has been raised since then and some experiments speak for an acceleration of the vital processes.

A stimulation effect of the beat frequency during irradiation of ciliated cells was nevertheless shown by Baldetorp et al. (1976) and Baldetorp (1977) and a stimulation of the activity of irradiated macrophages by Brandt and Baldetorp

(1979). These authors assumed that the increased activity was the result of a release of ATP, perhaps caused by a radiation damage of the membranes of the mitochondria resulting in increased permeability, or by a direct influence of the radiation on the cilia.

With increasing dose, the cilia within the irradiated area showed decreased frequency. The etiology of this phenomenon seems to be multifactorial. The edema probably plays a role, since the frequency diminished in correlation to the swelling of the epithelium. The increased number of blebs on the cilia at a higher dose may contribute to the result. A successive exhaustion of the ATP-synthesis indicates the same decrease of the frequency.

Single irradiation of the rabbit's trachea with 5 Gy, 30 Gy and 70 Gy, caused a reduction of the beat frequency (Fujiwara et al., 1972). At 5 Gy and 30 Gy, the ciliary beating reappeared within three hours. The activity was not restored after 70 Gy.

An experiment has been made concerning the immediate and early effects of irradiation

Table 2

Gy	Irradiated	Non-irradiated
2 - 10	++	+
12 - 20	+++	+

Table 2. Estimation of the number of goblet cells at different doses in relation to the ciliary cells of the irradiated and non-irradiated areas.

Figure 6. Irradiated rabbit trachea. 2 Gy 7 days after irradiation. Note the swollen tips of the cilia; the clustering of the cilia and the increased amount of blebs.

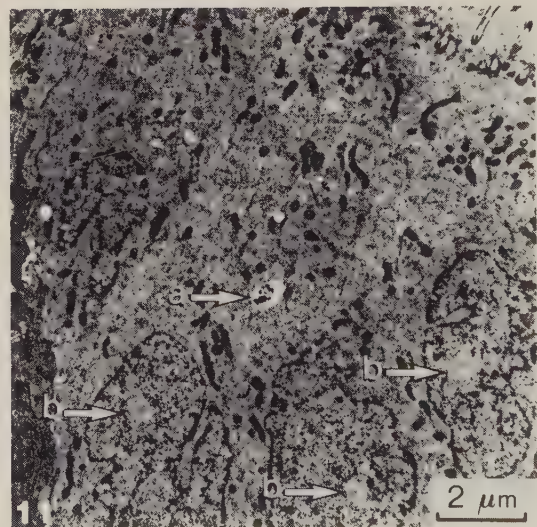
Figure 7. Irradiated rabbit trachea. 2 Gy 10 days after irradiation. Even here an increased amount of blebs can be found.

Figure 8. Irradiated rabbit trachea. 12 Gy 10 days after irradiation. Blebs scored to ++. Figure 9. Irradiated rabbit trachea. 20 Gy 1 day after irradiation. The amount of blebs are scored to +++.

Figure 10. SEM picture of an irradiated rabbit trachea. 2 Gy 7 days after irradiation. An increased amount of goblet cells (arrow) can be seen.

Figure 11. TEM picture of an irradiated rabbit trachea. 10 Gy 6 days after irradiation.

Radiation effects on ciliated cells



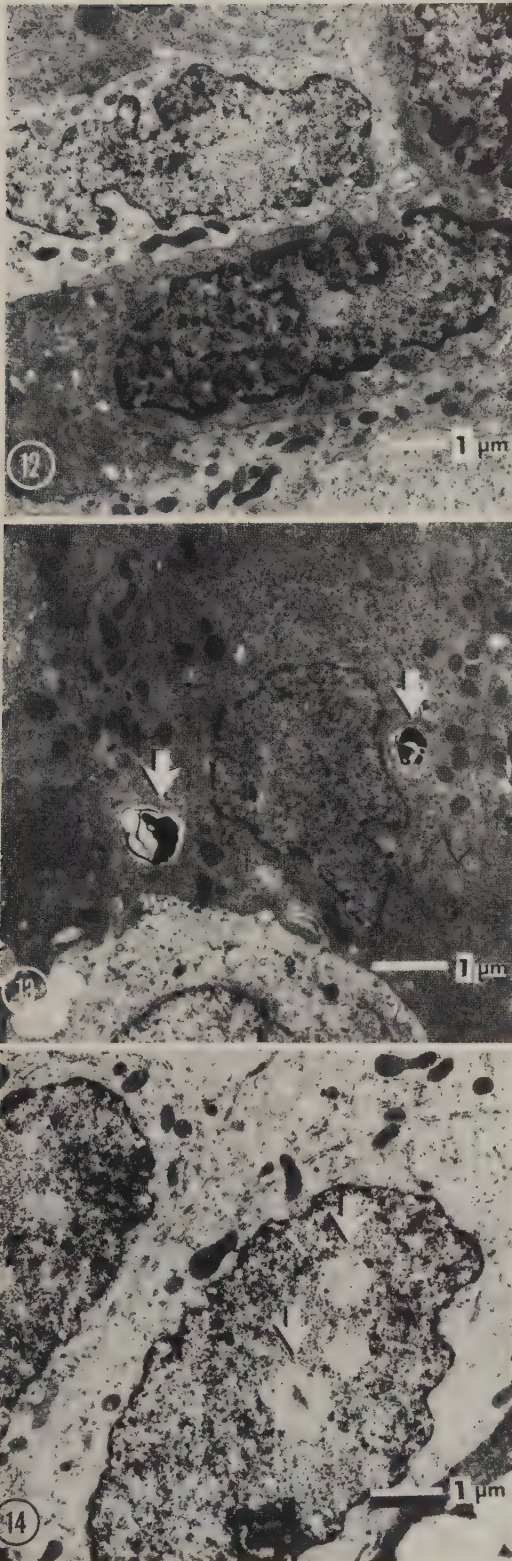


Figure 12. Multilobulated nuclei and increased number of nuclear pores. Figure 13. Heterophagic vacuoles (arrows). Figure 14. Inside the nuclei, zones with less density were seen.

(B. Baldetorp, personal communication). He treated a piece of the trachea with 10 Gy twice, with an interval of two hours. After the first irradiation he found an increase in the beat frequency of the cilia. After the second irradiation, there was no increase. He interprets this phenomenon as a reduction of the ATP after the first irradiation.

There are also other factors that can contribute to the reduction in ciliary beating e.g. the increased amount of goblet cells at higher doses. Goblet cells are stimulated by the irradiation and this is a well known phenomenon, especially in the intestine. (Friebert, 1980). It is possible that a rich amount of mucus lying heavily on the cilia may depress their activity. The mucus cannot, unfortunately be seen in today's preparations, but can possibly be visualised in the future by using a technique, newly-described where acrolein vapour is used as a fixation method. (Garland et al., 1982).

Knobs on the cilia are commonly explained as a fixation artefact, presumably caused by a change in the osmotic pressure of the buffer system. (Brunk et al., 1975, Ericsson et al., 1978). Weak regions on the cilia may be more sensitive and able to produce local swellings with a certain systemic development. (Dalen, 1982). This does not contradict the conception that external stimulus (heat, ionizing radiation) are able to produce blebs on the membrane of a cilium (von Mecklenburg et al., 1974, Baldetorp et al., 1976).

A great amount of ciliary blebs were found usually with increasing dose. These blebs are likely to constitute mechanical obstacles when the cilia perform their action, and many blebs may therefore cause a decrease in beat frequency.

The signs of increased metabolic activity inside the cell, e.g. change of the nuclear shape (Mirsky et al., 1961), as found within the high-dose range, may be attributed to repair processes

Conclusion

The experiments have thus far shown relations between physiological effects and morphological changes, but these are difficult to evaluate. At the present time we don't know enough about the biological events, to be able to express the radiobiological effect in a comprehensive formula.

Acknowledgements

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Discussion with Reviewers

B. Afzelius: One of the many effects of X-irradiation presumably is a heating of the tissue. Could the authors give a figure of the magnitude of the heat effect? Could this heating result in changes in the mucus, cell membrane or cytoplasm, which remain after termination of X-irradiation?

Are there any data of similar kind on the effects on the trachea by for instance microwaves or neutron bombardment?

Authors: In all the 100 rabbits the temperature was recorded with a thermistor in the animal immediately before the experiment (at pars membranacea) where the irradiation was made, and this was compared with the corresponding non-irradiated part. The greatest difference in one and the same animal that was measured came up to 1.8°C. There was no significant relationship between dose and temperature in these actual experiments. According to Mercke et al. (1974), see references, 2°C will give a difference of the beat frequency of about 50 beats/min. Possibly there is a thermal effect which can be measured ultrastructurally (Mecklenburg et al., 1974, see references). There is however, a relationship between the given dose and ultrastructural findings (e.g. blebs) on the cilia as measured by SEM and intracellular alterations as measured by TEM which supports the opinion that the temperature is not the predominant factor.

As far as we know, there are no similar experiments made by microwaves or neutron bombardment (= recording during the irradiation).

Reviewer III: Concerning the observations on ciliary activity in the controls (non-irradiated area), it is stated that the area observed is from the bifurcation of the bronchi. Which bronchi are being observed? Could the authors mean that they observed ciliary activity in main stem bronchi (which of course, result from bifurcation of the trachea)?

Two cm of the cranial part of the trachea were irradiated. What is the length of a rabbit trachea? How close was the posterior end of the irradiated zone to the tracheal bifurcation (and the start of the main stem bronchi, the site where the control samples were presumably taken from)?

Authors: The rabbit trachea has a length of about 7 cm. The unirradiated trachea has the same ciliary beat frequency down to the opening of the bronchi. In the more peripheral part of the bronchi the frequency is lower. From the 2 cm irradiated, the measuring point and area of sampling was in the cranial 1 cm part. The absorbed dose faded off sharply and 5 mm beyond the caudal part of the irradiated trachea no dose could be mea-

sured. The distance between this limit and the point from which recording was made and samples were taken was 40 mm.

Reviewer III: Please provide information about the procedures that were used when quantitative measurements were made on the histological material (edema, ciliary knobs, goblet cells), e.g. number of slides or photographs counted, how much variability was noted, and more exactly, how things were counted (for example, concerning ciliary knobs, were all knobs counted that could be seen in a selected area of a 12,000 x picture, but note that Figs. 8 and 9 are at a different magnification than Figs. 5-7; how was clustering of the blebs "taken into consideration". Were the specimens coded so that the counter did not know the identity of control and experimental preparations?

Authors: The edema was measured from 1-2 μ m thick sections as were the goblet cells. Six sections were cut from control and irradiated specimens in all 100 rabbits and each section had 115-120 ciliated cells. The goblet cells were calculated in relation to the cells bearing cilia.

We are aware of the difference in magnification in Figs. 8 and 9 as compared with Figs. 5-7 and the number of blebs was scored by the three authors. The changes were so prominent that it was possible immediately to differentiate between irradiated and non-irradiated trachea. The clustering of cilia was not taken into consideration concerning blebs.

Reviewer III: In Table 1 does the data in the irradiated column present observations at all time periods, e.g. day 1-10 after irradiation?
Authors: Yes.

Reviewer III: Since the density of goblet cells varies in different locations in the trachea, how can showing one SEM micrograph of an irradiated trachea (Fig. 10) be convincing evidence for more frequent goblet cells in irradiated trachea?

Authors: No, the SEM-picture alone, cannot be convincing. Therefore LM-calculation was used.

Reviewer III: In the TEM-studies, what procedures were used to evaluate the number of mitochondria and the conclusion that there is an increased number of mitochondria in irradiated cells?

Authors: This conclusion goes back to earlier experiments presented by Baldetorp *et al.* (1977), see references.

Reviewer III: Why is it believed that multilobulated nuclei is an indication of increased metabolic activity?

Authors: According to what is written about nuclei and physiological activity, it is believed that multilobulated nuclei appears to have an intense activity (Mirsky *et al.*, 1961), see references.

K.E. Carr: Are measurements available to support the statements that the number of mitochondria increases, that the number of nuclear pores increases and that there were more heterophagic vacuoles?

Authors: Yes. We are going to present this at a later date.

THE EFFECTS OF 10 Gy SINGLE-DOSE IRRADIATION ON THE CILIATED EPITHELIUM
MEASURED DURING AND ONE-TO-TEN DAYS FOLLOWING IRRADIATION.
A Comparative Physiological and Morphological Study.

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Abstract

Experiments recently done in this laboratory have shown that ionizing irradiation can result in an increase in the ciliary beat frequency of the trachea in vitro even during ongoing irradiation, implying an immediate stimulation of the physiological activity of the cells. This phenomenon is not fully understood and is by itself contradictory to general radiobiological concepts. Ultrastructural investigations on specimens taken immediately after irradiation (10 Gy) have, so far, not shown any changes. It has therefore seemed useful to extend the investigations by examining the effects of in vivo-irradiation (10 Gy) on rabbit trachea during the first 10 days after treatment. The following results have been obtained:

Phase I: On days 1 - 3 after irradiation the beat frequency showed a slight increase (10 %) within the irradiated part in comparison with a non-irradiated area of the trachea. No pronounced ultrastructural changes were found these days.

Phase II: On days 4 - 7 after irradiation the mucociliary activity was reduced in those places where it could be measured. The surface of the cilia assumed a generally destructed appearance with elongated cilia and disorganisation. TEM-pictures showed an increased amount of goblet cells and signs of membrane damage such as cytoplasmic extrusions at the apical end of the cells.

Phase III: On days 8 - 10 after irradiation a normalization of the tissue gradually took place with a beat frequency returning to normal. The ciliary surface showed a recovery process which also could be observed in the TEM-pictures.

KEY WORDS: irradiation, tracheal epithelium cilia, immediate effects, late effects, physiological activity, scanning electron microscopy, transmission electron microscopy.

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Introduction

The manifestation of the biological effects of ionizing irradiation is time-dependent, as has been demonstrated in both physiological and morphological studies of these effects. Earlier investigations would seem to indicate that modern methods detect the physiological effects earlier and with greater facility than is the case when the morphological effects are studied. Baldetorp et al (1976) and Baldetorp (1977) thus found an increase of the mucociliary wave-frequency in the tracheal ciliated cells of the rabbit coming as a stimulatory effect during irradiation by photons at different dose rates. This effect was usually detectable at an accumulated dose of 1.5 Gy, but was not accompanied by any morphological changes which could be seen with electron microscopy.

Ogi (1959) studied the effects of beta-irradiation on the ciliated cells after completion of irradiation. He found a decrease of the beat frequency, but also noted recovery after 2 - 3 hours in the dose range of 7 - 14 Gy.

Fujiwara et al (1972) found principally the same effects after irradiation with photons. Morphological studies were, however, not performed. The asserted stimulatory effect of irradiation is still a matter of dispute, and the experiments reported in this paper have been conducted in order to further investigate the seemingly contradictory findings of others, as described above, in the hope of finding more information, and in order to compare the immediate and later effects of irradiation.

In our study, the trachea of the rabbit received 10 Gy in vitro, and the beat-frequency was measured during this irradiation. Electron microscopy was made on preparations immediately following irradiation. The results have been compared with those obtained from experiments where the trachea of the rabbit was irradiated with 10 Gy and measurements corresponding to those of the present study

made during ten consecutive days after irradiation.

Material and Methods

"Immediate Effects": Irradiation in vitro

Forty rabbits were used for the examinations. They were sacrificed by a blow on the skull in order to avoid side-effects, whereupon 70 mm of the trachea was extirpated and divided in two equal parts. They were then stretched to about 20 per cent more than the contracted state, and placed in the experimental chamber. One specimen was placed in a lead-shielded box, and the other mounted for measuring the beat-frequency. The details have been described earlier (Håkansson and Toremalm, 1965; Baldetorp et al. 1976), since the well-known light-beam reflex recording was used. Ten Gy were given to the specimen, and immediately after the irradiation, sections were cut both from the irradiated trachea and the control in the lead-shielded box, which were studied with Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). In order to obtain energy quantities which could be used for comparison with the "late effects" (1 to 10 days after treatment), the in vitro-irradiation was performed with two different qualities:

1. X-ray, generated at 50 kV, 2 mA, filter: 0.5 mm Al, Half Value Layer: 0.05 mm Al, focus-object distance: 80 mm. The dose rate was 0.05 Gy/s. A Philips contact therapy apparatus was used. Each tracheal specimen received 10 Gy. Twenty rabbits were used.

2. Gamma-radiation from ^{60}Co , source diameter: 15 mm, source to front of diaphragm distance: 300 mm, source-object distance: 610 mm and field size: 130 mm x 130 mm at 610 mm source-distance. The dose rate was 0.017 Gy/s. A Siemens Gammatron III unit with multiplane collimator was used. Twenty rabbits were used.

Dosimetry-measurements were carried out with thermoluminescent dosimeters (TLD) at the place in the experimental chamber where the centre of the tracheal mucous membrane was lying during exposition.

"Late Effects": Irradiation in vivo

Ten rabbits were used and the examination was performed as described above. The first measurements were made one day after irradiation on one of the rabbits, and the others were investigated on the following consecutive days. The last rabbit was examined on day 10 after irradiation.

Irradiation was performed by a conventional Siemens X-ray unit using 160 kV and with a HVL: 4 mm Al. Skin-Source Distance (SSD): 50 cm. Dose-rate = 0.013

Gy/s. Before the irradiation, the animals had been rendered unconscious by intraperitoneal injection of pentobarbital (40 mg/kg body-weight). The rabbits were laid on their back, and the upper part of the ciliated trachea (20 mm) was irradiated. The caudal limit of the treated area was lead-shielded, and the direction of the beam was arranged in a cranial angle, in order to strike the caudal part along a cartilage arc. The spatial distribution between the irradiated part and the control part was 40 mm. The two sections were placed in the experimental chamber as above, and the beat-frequency was recorded from each preparation. Immediately prior to measurement, sections were cut for SEM- and TEM-examinations.

Preparation for SEM

The pieces for SEM-examination were not rinsed, but were placed directly in 2.5 % glutaraldehyde for fixation during 12 hours (in 0.15 M cacodylate buffer). The pH of the solution was 7.3. They were then transferred into the same buffer, and were later osmium-fixed in 1 % osmium tetroxide in 0.15 M cacodylate buffer for two hours.

Dehydration took place with graded series of ethanol, after which the preparations were transferred to Freon TF 618. The specimens were later dried according to the critical point method in Balzer's 000 Critical Point Dryer. They were finally sputter-coated with gold plus palladium in a Polaron SEM-coating unit E 5000. Then they were examined in a Cambridge Stereoscan Mark II A or a Zeiss Nanolab Electron Microscope. The microscopes were operated at 20 kV.

Preparation for TEM

The samples were fixed as for the SEM-preparations and also in 1 % OsO_4 in phosphate buffer (pH 7.4) for two hours, rinsed in 0.15 M cacodylate buffer, dehydrated in alcohol and embedded in Vestopal W or Epon. Sections of 1 μm thickness were cut out on an LKB-Ultratome, stained with toluidine blue and examined in a light microscope. Ultrathin sections were cut out and contrasted with lead citrate and uranyl acetate, or en bloc with 0.5 % uranyl acetate. A Zeiss EM 10 Electron Microscope was used.

Statistical Analysis

Statistical analyses were done with regression analysis using the package "Minitab".

Results

Immediate Effects

In the normal trachea, the beat-frequency was 987 ± 30 beats/min. In those specimens irradiated with 50 kV X-rays, the frequency increased up to about 25 % at an accumulated dose of 1.5 Gy (Fig. 1, partly extracted from Baldetorp, (1977)).

The effects of 10 Gy single-dose irradiation

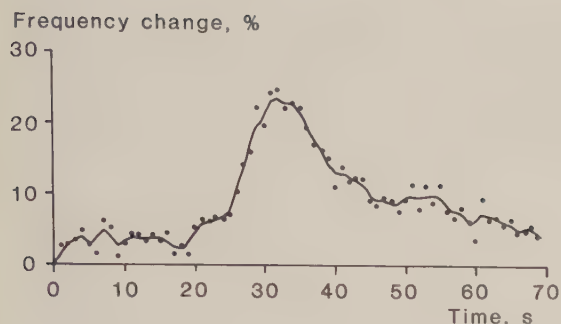


Figure 1. The change in the beat-frequency during irradiation. 50 kV, dose rate: 0.05 Gy/s. Each point represents the mean value for 20 rabbits.

It then decreased to a level just above the normal value after a dose of 3.0 Gy. ⁶⁰Co-irradiation caused a corresponding increase (Fig.2) up to 22 % following an absorbed dose of 1.3 Gy. The increment lasted longer (30 s), and declined very slowly.

Morphology: Specimens taken immediately after the irradiation were investigated by SEM and TEM. No deviations from the non-irradiated part (cf description below) were seen.

Late Effects

The beat frequency of the irradiated part could be measured over the whole surface the first days after irradiation. When measured second-by-second, it varied within a range of 4 Hz as has generally been found in earlier experiments. Figure 3 shows the mean value in beats per minute from each rabbit from day 1 to day 10 after irradiation, related to the mean value as measured in the non-irradiated area. It is noticed that, especially during the first days, the quotient has a higher value than 1.0, which would indicate a stimulation process.

It was, however noticed that, during days 3 to 8, only part of the mucous membrane could be used for determining the frequency; the surface seemed inactive, and reached its lowest point on days 4 to 6, when only separate small areas were active. India ink was placed on the surface, and it could then be established

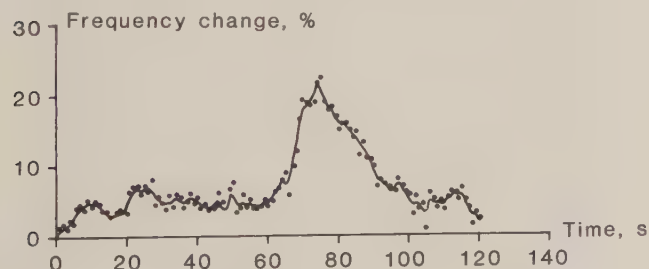


Figure 2. The change in beat-frequency during irradiation. ⁶⁰Co, dose rate 0.017 Gy/s. Each point represents the mean value for 20 rabbits.

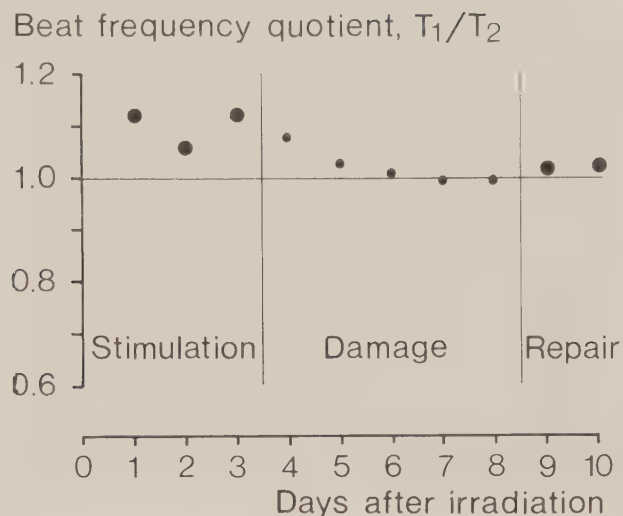


Figure 3. The ciliary beat-frequency within the irradiated part of the trachea (T₁) in relation to the beat-frequency in the non-irradiated part of the trachea (T₂). One point is the experimental data from one individual rabbit, and the ten results were obtained from the first day after irradiation to day 10. ● = normal, • = abnormal surface.

that, on certain small spots, the particles moved only in circles, while as a whole the mucous carpet was not moved as in the normal cases. The frequencies of the active cilia were, however, recorded, and the results are shown in Fig.3, where the large points represent recording from a surface with normal transport capacity and the small points are recording from a surface with reduced transport capacity.

The amplitude of the oscillations from these cilia groups was, furthermore, lower, as compared with those recorded on the non-irradiated trachea, or those recorded one or two days after irradiation. This may indicate a reduced ciliary efficiency in regard to their strokes. It is nevertheless interesting that some groups of cilia showed a frequency slightly above, or similar to, that recorded (in the same way) from the non-irradiated part. The curve is interesting *per se*, even if it does not reflect the true mucociliary activity of the mucous membrane. This condition is more clearly visualized in the SEM- and TEM-recordings.

Morphology: (Scanning Electron Microscopy). In the non-irradiated material, a smooth carpet of cilia with an even structure and only a few blebs were seen, as well as a small number of goblet cells coming up to the surface with a coating

of small microvilli. In the irradiated material, changes were noted from day 1 to day 10. To facilitate summing up the findings, it seemed preferable to use a scoring system, where the different pictures were judged by four persons, independently of each other. This system employed units from 0.0 to 4.0, the last figure describing the figure with the greatest amount of injury. To obtain a more gliding scale, and perhaps a more refined judgement, the 0.5-fraction was put between the numbers.

The Score System

The following definitions were worked out for the respective values used:
0.0 (Fig.4 a,b): Normal surface with clean cilia having a minimum of blebs (5 - 24 on a defined area), and with regular arrangement. In rare cases, with visible goblet cells having intact microvilli.

No mucus granula or fibrin.
1.0 (Fig.5 a,b): The cilia have a regular pattern which is constant, as can be seen on a 1000 x picture. Blebs occur especially on the tips of the cilia, but also on the sides (to a number of 25 - 49 on a defined area at 5000 x magnification. Only solitary mucus granula are to be found on the surface. The goblet cells have normal microvilli.

2.0 (Fig.6 a,b): Blebs to a number of 50 to 74 on a defined area at 5000 x magnification. A rich amount of goblet cells with their content protruding on the surface having disintegrated and shortened microvilli. Some of the goblet cells have emptied their contents and mucus granula are seen on the surface. Many cilia are seen adhering to one another.

3.0 (Fig.7 a,b): Blebs occur to a number of 75 - 99 on a defined area at 5000 x magnification. The cilia are arranged in a regular pattern. Thick masses of emptied goblet cells with mucus granula are spread over the surface, the remaining goblet cells having almost no microvilli. There is a coating of detritus.

4.0 (Fig.8 a,b): Blebs are found to a number of 100 or more on a defined area at 5000 x magnification. The cilia are in a disorderly pattern, and have disintegrated. The goblet cells are emptied. The surface is covered with detritus and probably with fibrin. A few cilia with intact structure can be seen here and there. Figure 9 shows the results of the evaluation by this method.

Each point represents the mean value of the judgement of the four persons. The curve was statistically analyzed, and the results showed a significant difference between the appearance of the surface during the first three days and those between days 4 and 7. Day 8 and the follow-

Figure 4a. Score 0.0. Normal, ciliated trachea.

Figure 4b. Enlargement from Fig. 4a. Note the clean cilia.

Figure 5a. Score 1.0. The cilia appear normal. At the arrow a goblet cell is emptying its content with normal microvilli on the surface.

Figure 5b. Score 1.0. Enlargement from Fig. 5a.

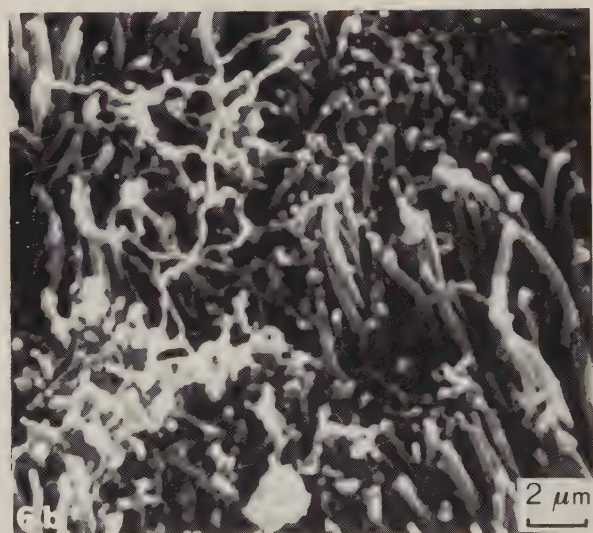
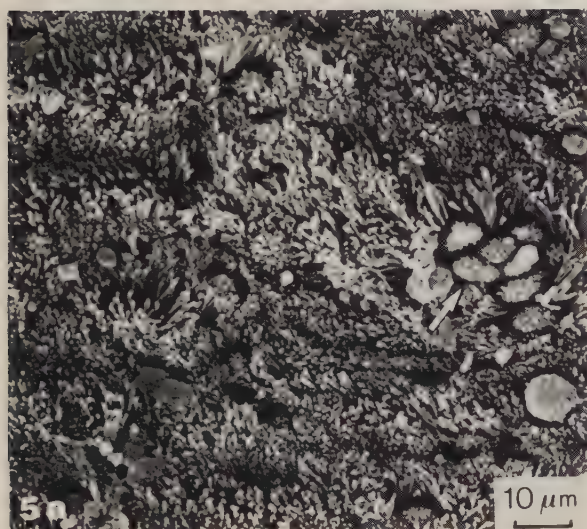
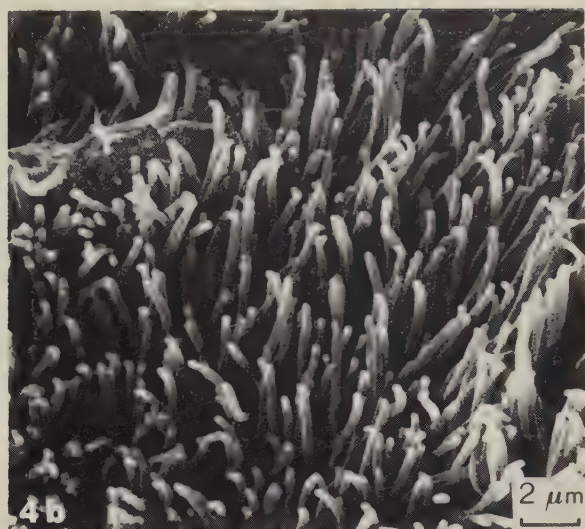
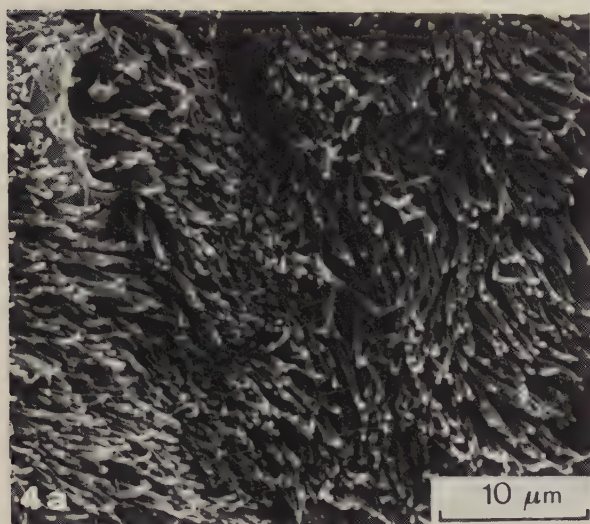
Figure 6a. Score 2.0. At arrow: shortened microvilli on the contents of the goblet cell.

Figure 6b. Score 2.0. Enlargement from Fig. 6a. Rich amount of detritus.

ing days also differed from the values of days 4 - 7 ($p < 0.002$). The variation as a function of time seemed to be both linear and quadratic. In order to see if different structures followed the general impression of the consecutive pictures, an attempt to apply more objective measurements was made by measuring the thickness of the cilia on the pictures with 5000X magnification. This was also done by four persons independently of each other. Thirty cilia from each specimen were calculated. Bearing in mind that the diameter of the cilia varies considerably at the tips, measurements were only made where there was even thickness. In SEM, the diameter of the cilia was in the range of 0.3 - 0.4 μm , and therefore not consistent with the accepted value. The SEM-measurements are only relative and the contribution of the gold-palladium film is included. The variation of the ciliary diameter in relation to time was, however, statistically significant ($p < 0.002$), with both a linear and quadratic effect (Fig. 10). The exact value was then measured on TEM, which showed the same pattern (Fig. 10). Measurements from days 1, 5 and 10 are presented. The diameter is here within the accepted values of 0.2 - 0.3 μm , with the lowest value on day 5 after irradiation.

Transmission Electron Microscopy (Non-irradiated material): The normal trachea showed the characteristic layer of basal cells, ciliated cells and goblet cells above a supporting structure of collagen fibres. The cilia had the normal length of about 7 μm , and a thickness of 0.2 - 0.3 μm . Rich amount of microvilli from the

The effects of 10 Gy single-dose irradiation



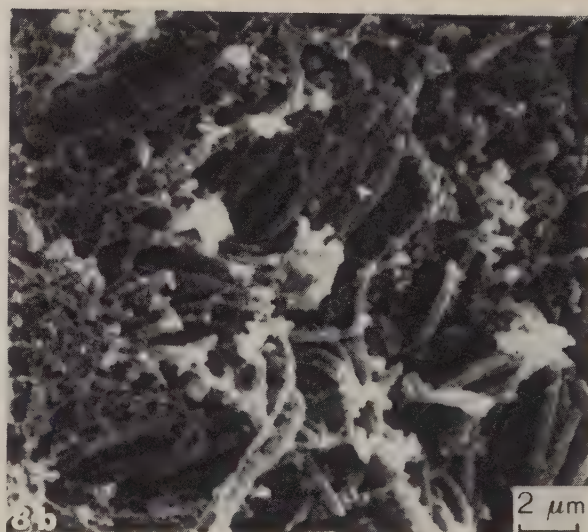
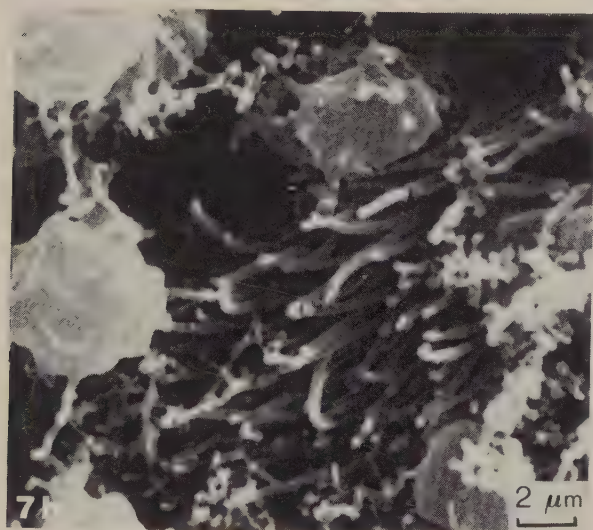
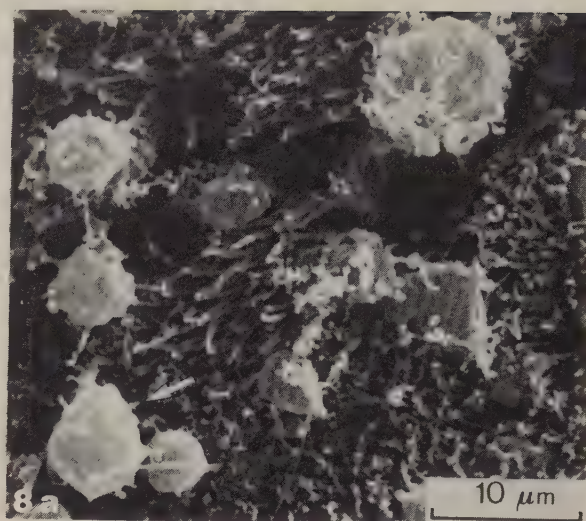


Figure 7a. Score 3.0. Granula from emptied goblet cells (arrow). Shortened microvilli.

Figure 7b. Enlargement from Fig. 7a.

Figure 8a. Score 4.0. Heavily injured surface.

Figure 8b. Enlargement from Fig. 8a.

surface of the ciliated cells were found between the cilia. They had a length of 3 - 4 μm . Inside the ciliated cell, the nucleus was in the basal part. The usual configuration of the nucleus was oval or circular, with no invaginations. Endoplasmatic reticulum with the usual smooth appearance was found, as well as free ribosomes. The goblet cells between the ciliated cells had vesicles with mucous granula. Some of the cells had come to the surface of the epithelium layer, where small microvilli were seen on the membrane. These microvilli had a length of 0.4 - 1.0 μm . Some of the goblet cells had emptied their contents, as apocrine cells and only remnants of the cell bodies were interspaced between the ciliated cells. Mitochondria were seen both in the

basal cells, the goblet cells and in the ciliated cells. They had their usual appearance, being of oval or elongated form, with unbroken cristae.

Irradiated material: On the basis of the findings with the score system, and the results from measuring the diameters of the cilia, it seemed preferable to look at the TEM-pictures as representing three time periods after irradiation with 10 Gy: Phase I; days 1 - 3 (called the stimulation phase), Phase II; days 4 - 7 (the damage phase), and after day 8 Phase III (the repair phase).

Phase I (Stimulation): Many of the cilia gave an impression of having a greater distance between the microtubules and the outer membrane. The 9+2 structure was clearly seen, and a small bleb was found

The effects of 10 Gy single-dose irradiation

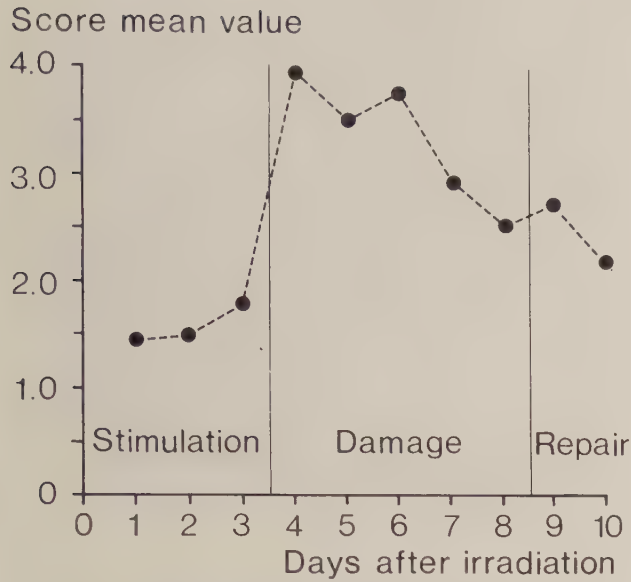


Figure 9. Mean value for the scoring of the SEM-pictures.

at the tips of some cilia. The mitochondria were swollen, and some of them had broken cristae. The form of the nuclei did not have its usual even, rounded or oval structure. In some cells, a ductal invagination or lobulation of the nucleus was noted. The chromatin was distributed within the whole nucleus, but with a tendency to accumulate at the membrane where the pores of nuclei could be detected. The goblet cells occurred more frequently than is normal, and showed a distinct and perceptible endoplasmatic reticulum.

Phase II (Damage): The cilia were not swollen as in the first phase, but had blebs on their sides. The 9+2 pattern seemed unchanged. The mitochondria were still swollen, and broken cristae could be seen. There was also a tendency to an accumulation beneath the basal bodies of the ciliated cells. The amount of goblet cells were even more increased. From the apical part of some ciliated cells, cytoplasmic evaginations or protrusions contained amorphous material and it was difficult to observe any distinct membrane (section effect?), but it could be imagined on certain small spots (Fig. 11). Endoplasmatic reticulum and Golgi complex were clearly seen, especially in the goblet cells, which were found to have large mucous vesicles with small granula (Fig. 12). The chromatin was distributed as described earlier, but small areas with less density were seen. The nucleolus were clearly visualized (Fig. 13a). The nucleus membrane was often lobulated and showed a rich amount of nuclear pores (Fig. 13b). During this second phase, many heterophagic vacuoles were seen.

Phase III (Repair): The cilia had an even membrane, and cross-cut sections showed

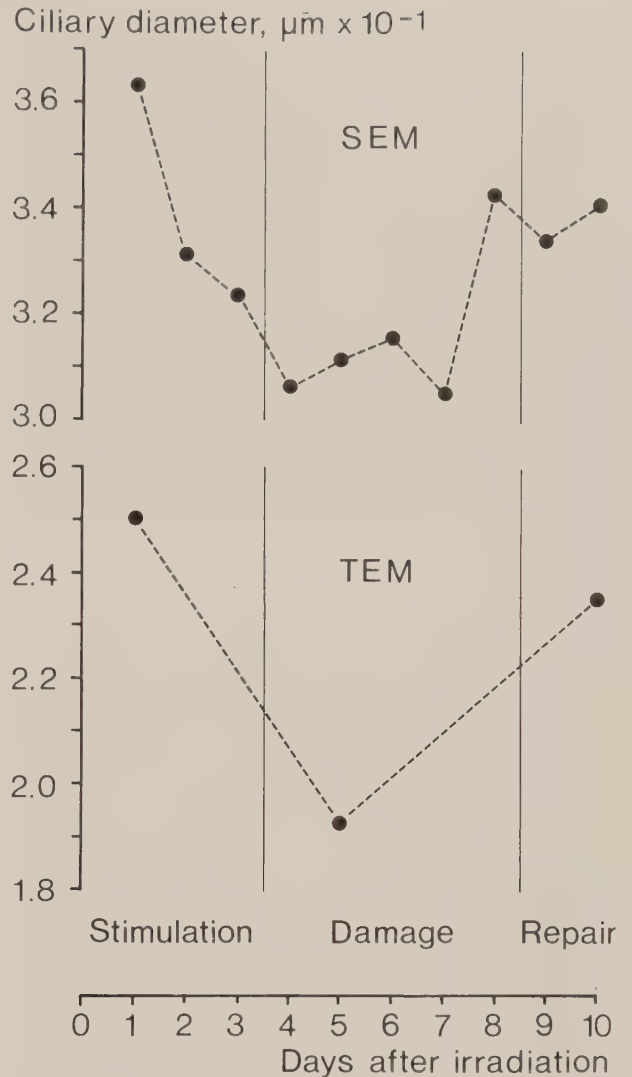


Figure 10. Upper picture: Diameter of the cilia as measured from the SEM-pictures. Each point represents the mean value of 30 measurements.

Lower picture: Diameter of the cilia measured from TEM-pictures.

the usual 9+2 pattern. The distance between the microtubules and the outer membrane was as in the control material. The mitochondria also showed a distribution similar to that of the controls. During the early stage of this phase, some nuclei had a lobulated form and showed some areas with less density. The distribution of chromatin had not changed. Ribosomes and endoplasmatic reticulum were clearly seen in the cytoplasm. The number of goblet cells was likewise increased, and heterophagic vacuoles were also seen.

As a whole, these phases overlap each other, which indicates dynamic processes that take part at different sites in the ciliated cells.

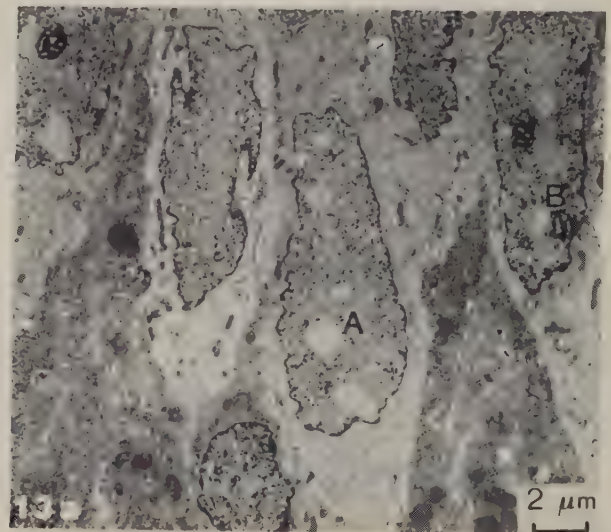
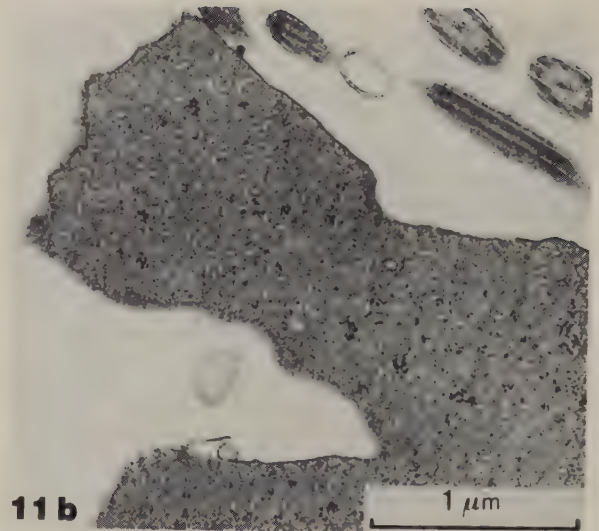
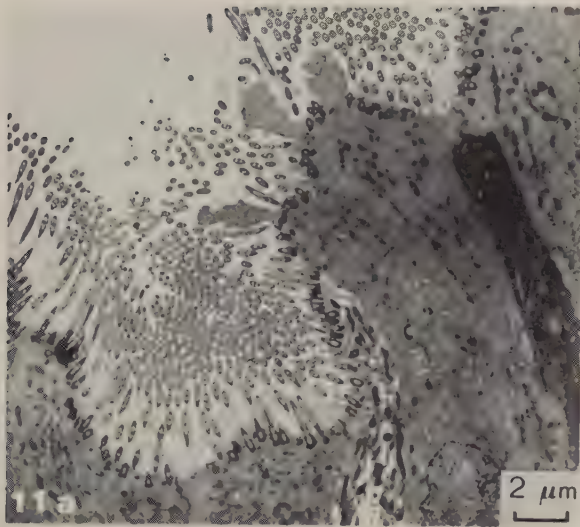
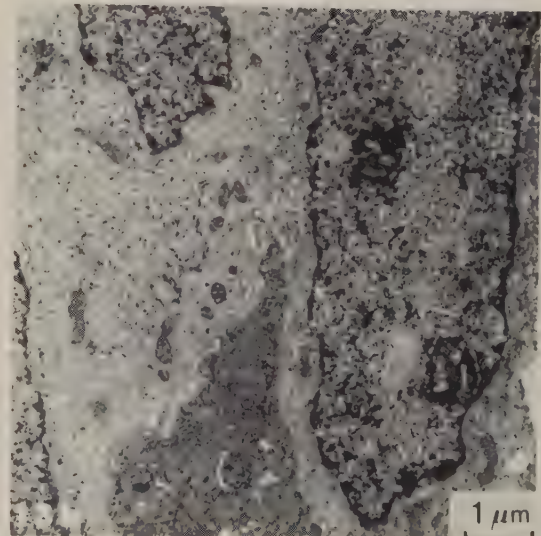


Figure 11a. Arrows: Cytoplasm protruding from the ciliated cell.

Figure 11b. Higher magnification of the extrusions seen in Fig. 11a. Note the existence of a membrane on the right side of the protrusion.

Figure 12. TEM-picture of the irradiated ciliary epithelium. Note the large mucous vesicles in the goblet cells.

Figure 13a.b. TEM-pictures. Note the multilobulated nuclei, the zones within the nucleus with low density (A), and the marked nucleoles (B).



Discussion

In order to be able to accurately compare the "immediate" and "late" (1 - 10 days after treatment) effects of irradiation, it is necessary to first define these concepts. Immediate effects are here considered to be those which can be measured during irradiation, while late effects are those which are detectable one-to-ten days following irradiation. Concerning the immediate effects, they are induced by a radiation energy varying from low X-ray irradiation at 50 kV to high-energetic irradiation using 1.25 MeV photon irradiation. The Compton effect can be assumed to dominate at both of these levels of energy, as well as that energy used in the late-effect experiments, i.e. X-ray irradiation at 160 kV. The trials are thus acceptable from the standpoint of irradiative energy. The differences in dose rate can also be proved: 0.05 Gy/s was used in the attempts with 50 kV irradiation, and 0.017 Gy/s was utilized in the trials with ^{60}Co . When 160 kV X-rays were used, there was a dose rate of 0.013 Gy/s.

Immediate effects

The increase in frequency seen during irradiation is rather confusing and quite difficult to explain. This initial effect is nonetheless indisputable. It is also confirmed in experiments with lower ciliated organisms. As a matter of fact, it was proposed as early as 1903 by Bohn. The general assumption today is that adenosine triphosphate (ATP) serves as energy for the ciliary motility, and that the concentration determines the beat frequency (Satir, 1974). According to the "enzyme release theory" proposed by Bacq and Alexander (1961) as an effect caused by irradiation, the increased speed of ciliary movement may be explained by a release of ATPase, and the immediate effect will thus take place within the cilium itself. This increase is not followed by a change of the electrophysiological activity (Baldetorp et al. 1983). Immediately after the irradiation, both Ogi (1959) and Fujiwara et al (1972) found that the beat-frequency decreases. Although Ogi used beta-particles and Fujiwara et al, photons, their results were principally the same: a decrease followed by a return to a normal value within a period of 2 - 3 hours (dose range: 5 - 30 Gy).

The decrease of activity during the late phase of irradiation may be explained by a reduced pool of ATP in the cilia and a lack of newly synthesized ATP from the mitochondria. It is known that a resynthesis of ATP can take place within the cilia from available nucleosides, by means of the action of phosphotransferase on adenosine diphosphate (ADP) (Saavedra and

Renaud, 1975). Nothing is known, however, about the length of the time-factor of this resynthesis.

No morphological changes could be observed on the preparations taken immediately after irradiation. Ogi (1959) found no such changes, either, in this dose-range. His findings in this respect were confirmed by Baldetorp (1977), who found that it requires doses above 30 Gy to produce changes detectable with electron-microscopy.

Late effects

Phase I (Stimulation): Recordings of the beat-frequency showed that it was higher than normal during days 1 - 3 following irradiation. This is interpreted as indicating that the beat-frequency is stimulated by irradiation. It is, however, not logical that this phenomenon can be explained in the same way as the stimulation observed during irradiation. ATP is delivered by the mitochondria, and if a true stimulation exists, caused by irradiation, an increased synthesis of ATP may take place, thus increasing ciliary activity, as measured during days 1 - 3.

Another possibility is that there exists a feed-back mechanism from the cilia to the mitochondria, and that if irradiation has caused depletion of the ATP, this gives a signal to the mitochondria to synthesize more ATP.

It is interesting to note the difference between the rates of increase of the activity found in the studies of the early effects and those found in the studies of the late effects. There is 20 - 25% increase of activity during irradiation, but the increase is only 10% during days 1 - 3. TEM-examination of the irradiated *in vivo* cells reveals that they contain mitochondria with swollen membranes, which indicates that they are affected by irradiation. It is at present not possible to say if this stimulation seen on days 1 - 3 is due to damage of the membrane, with an eventual leakage of ATP. During the first three days, there are no pronounced ultrastructural changes. The conclusion must be drawn that these first three days are a phase of stimulation, although the background for this is not known.

Phase II (Damage): This first phase passes into a phase where the mucociliary activity is reduced in those places where it can be measured. At the same time, the surface of the cilia assumes a generally destructed appearance, with elongated cilia and disorganisation. In the later part of this phase, most of the ciliary cells have been put out of action, corresponding to the appearance of the ciliary surface seen by SEM. The later effects of irradiation are better evaluated by SEM-study than by TEM-examination.

The second phase, i.e. days 4 - 7, after irradiation reveals damage to the structure: there is a disintegration on the surface, and the diameter of the cilia is diminished. The observed failure of the mucous carpet to move can also be explained by mechanical action on the ciliary crown (Dirksen and Satir, 1972). This can be assumed to be indicated by the findings of the increased numbers of goblet cells, which is most dominating on days 4 - 7 following irradiation. If these cells empty their contents, the carpet covering the ciliary cells ought to be of higher viscosity.

The most likely explanation is that both stimulating and damaging effects interact during this period.

Phase III (Repair): During this period, a normalization of the tissue gradually took place, perhaps because the trachea had been in the host for up to 10 days, and the prerequisites for the above-mentioned assumption are then most favourable. It is also consistent with the increase in diameter of the cilia, and a scoring of lower values when judging the SEM-pictures. The beat-frequency tends to be the same as in the nonirradiated area of the tracheal mucous membrane. The TEM-pictures also show this normalization process.

The third phase may therefore be regarded as the "period of main repair". This does not exclude, of course, the possibility that reparative processes begin to work at a much earlier phase. The role of blebs on the cilia is difficult to explain. Their origin may be spots of weakness of the membrane of the cilium (Dalen, 1983).

Conclusion

Finally our present knowledge of the effects of 10 Gy irradiation given to the tracheal epithelium can be summarized in figure (14).

Acknowledgements

We wish to express our gratitude to Miss Marianne Palmegren, who has done all the preparations for SEM- and TEM-examinations, at the oncology-EM laboratory. The institute of Zoology, University of Lund, Sweden, has kindly provided the facilities for scanning and transmission electron microscopy. The investigation was supported by grants from John and Augusta Persson's Foundation, and from the Medical Faculty, University of Lund.

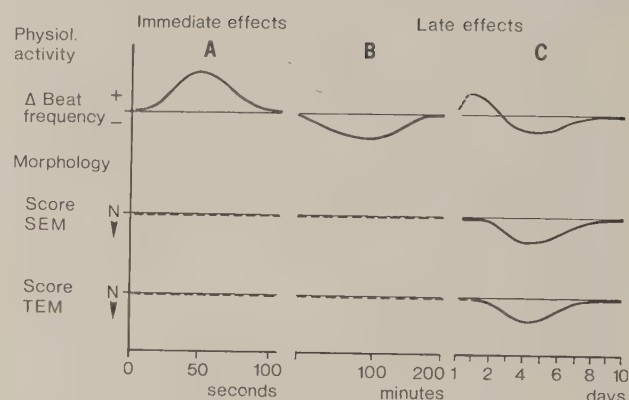


Figure 14. A summary of the events of the ciliated tracheal epithelium during and after irradiation with 10 Gy.

A: Baldetorp (1977) and Baldetorp (this paper).

B: Ogi (1959); Fujiwara et al (1972).

C: Albertsson (this paper).

+, - = Higher or lower activity, respectively, than normal.

N = Normal structure.

▼ = Deviations from the normal structure in arbitrary units.

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Discussion with Reviewers

B. Afzelius: Tracheal cilia are mucous-propelling ones and treatments that give an increased beat frequency may not necessarily be due to effects on the cilia themselves. The primary effect may be on the amount and viscosity of mucous. Have these factors been checked?

Authors: The amount of mucous has not been checked but we have tried to measure the viscosity or the tenacity, because the mucous is not a Newtonian liquid. Commercial apparatus cannot be obtained because it is not possible to get more than a few µl of mucous. We have therefore developed an apparatus for the purpose: Håkansson and Toremalm: A method for determining the viscoelasticity tracheobronchial secretions. *Am. Rev. Resp. Dis.* 1970. 102, 1, 42-47. The investigations have not shown a change in the viscoelasticity due to irradiation.

B. Afzelius: Increased frequency occurs at higher temperature, e.g. at fever. Did the animals have fever after irradiation?

Authors: No, and this is because the size of the irradiation field is small (10 x 20 mm). We have measured the temperature beneath the trachea and there is an increase in the temperature of 1.8°C at its maximum. This corresponds to an increase of the beat frequency of less than 25 beats/min.

B. Afzelius: There is a subgroup of the immotile-cilia syndrome in which the cilia have a hyperfrequent beat (>13 Hz) and which by Pedersen et al. *Europ. J. Resp. Dis.* 64 Suppl 127, 78-90, 1983 is called primary ciliary dyskinesia.

Although the cilia in this subset of the syndrome beat rapidly the beat is inefficient. Do the authors have any information on whether the cilia in the "stimulatory phase" of their irradiation experiment do useful work? If not, the term "stimulation phase" may be misleading.

Authors: When Pedersen et al. find ciliary dyskinesia they claim that the recording of the beats gives irregular wave pattern. In our case during the stimulatory phase the waves were quite regular.

B. Afzelius: On days 4 - 7 post-irradiation ciliary transport goes in circles rather than straight as it should for useful work. How about the orientation in the stimulation phase?

Orientation can be evaluated in the living state and on ultrathin sections and also by noting the orientation of the ciliary tips in the SEM. Ciliary tip is bent in the same direction as the effective stroke (Veerman et al. *Pediatrics* 65, 698-702, 1980).

Authors: During the "stimulation phase" the india ink flowed cranially with the mucous. Unfortunately we have not looked upon the orientation on the sections. We will do this.

B. Afzelius: How do the authors visualize cilia that can beat with the dynein detached from the microtubules? If this is the idea of the "enzyme release theory" then the current concepts of ciliary motility have to be revised.

H. Dalen: The authors have suggested that the changes in the ciliary beat-frequency following irradiation may be coupled with the release of dynein. If so, have any morphological alterations of the dynein arms been observed in the present study?

Authors: We did not observe any morphological alterations in the dynein arms of the cilia. We think that the irradiation increases the access to ATP. In what way we do not know. According to Brokaw (*Mechanics and Energetics of cilia*, *Am. Rev. Resp. Dis.* 1966. 93 No 3 part 2) the supply of ATP in short cilia "will be more than adequate". Therefore we think that the irradiation is capable of giving more energy to the system, at least during the first seconds of treatment.

B. Afzelius: Resynthesis of ATP by the $2\text{ADP} \rightarrow \text{ATP} + \text{AMP}$ reaction will not suffice for keeping an ATP-requiring process going for long, as the store of ATP always is quite small due to its relative toxicity. Any comments?

Authors: We cannot refute your statement and are not able to answer your question.

K.E. Carr: Can any figures be given of the changes in amplitude associated with ciliary beat?

K.E. Carr: Were there any changes in the basal lamina of the epithelium?

Authors: Probably yes. Ten Gy, however, seems to be a border line between detectable and undetectable changes. We want to come back to your question when we have done various single-dose experiments.

R.F. de Estable-Puig: Why were different irradiation protocols utilized for the in vitro and in vivo experiments?

Authors: The experiments where a stimulation effect was noted were made in vitro with 50 kV irradiation. This energy cannot be used on a living animal since most of the energy is absorbed before the irradiation reaches the dorsal part of the trachea. At this time our experiments comprise energies up to 4 MeV photons and electrons, different dose rates and different RBE-factors.

R.F. de Estable-Puig: Which are the features which allow you the identification in SEM of empty goblet cells?

Authors: In this laboratory the emptying of goblet cells from the small intestine has been worked out carefully (L. Friberg: Effects of irradiation on the small intestine of the rat. A SEM Study. Thesis Berlings, Arlöv, Sweden, 1980). There are similar findings in the actual system with small "sacs" with microvilli coming up to the surface as a result of the irradiation.

R.F. de Estable-Puig: In the first phase of the in vitro experiments, is the stimulation effect observed on all the ciliated surface or only in groups of cilia?

Authors: On all the ciliated surface.

R.F. de Estable-Puig: Which was the delay from the end of irradiation until irradiated and control sections were placed in the chamber, previous to the recording of the beatings?

Authors: 1 st rabbit 24 hrs. 2 nd rabbit 48 hrs after irradiation etc. The last rabbit was examined 240 hrs after.

H. Dalen: What is the explanation for the cellular mechanism leading to increased ciliary bleb formation after irradiation?

Authors: In fact we do not know. We see the same inside configuration of these blebs as you have published earlier. It is strange that irradiation can have this effect. We do not exclude other causes.

RTO 00086

Scanning electron microscopy and transmission electron microscopy of the ciliated cells of the trachea of the rabbit treated with misonidazole alone and in combination with ionizing radiation

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Key words: Ciliary epithelium; Irradiation; Misonidazole; Light microscopy; Scanning electron microscopy; Transmission electron microscopy

Summary

The trachea is often located in the treatment volume when irradiating malignant tumours in the thorax. In order to evaluate possible synergism between misonidazole and irradiation on this tissue, the following studies were made.

Fifty rabbits were treated with daily injections of 100 mg misonidazole given i.p. on consecutive days from 1 to 10 days. Morphological investigations of the trachea were made with scanning electron microscopy (SEM), transmission electron microscopy (TEM) and light microscopy (LM). Physiological examinations were performed with recording of the ciliary beat frequency. The results were compared with those from a group of 100 rabbits given misonidazole in a similar manner and exposed to irradiation (2 Gy) 15-30 min after each injection. Ten rabbits were used as controls. The results are compared to the effect of fractionated irradiation alone with 2 Gy/day.

Fractionated irradiation of the ciliary epithelium in the trachea of the rabbit has shown dose-dependent physiological and morphological effects. Misonidazole potentiates these effects of radiation with a more pronounced change of the ciliary beat frequency and an increased metabolic activity as could be visualized on TEM. The combination of drug and irradiation also induced a hyperplasia of the ciliary epithelium. Misonidazole itself had no effect on the ciliary beat frequency, but caused a hypoplasia of the ciliary epithelium.

Introduction

Hypoxia seems to be characteristic feature of most tumour tissues. Since the cell-killing effect of ion-

izing irradiation is 3 times greater on well-oxygenized cells, hypoxia has long been thought to be a limiting factor for the ultimate cure of some tumours by radiation therapy. For this reason, radio-

biologists and radiotherapists have long been trying to find drugs that imitate the effects of oxygen. The nitroimidazoles have proved to be the most efficient group of substances in this respect, particularly metronidazole and misonidazole [6,31].

Originally, the mechanisms of sensitization of these drugs have been described both as a fast free radical reaction, imitating the effect of oxygen to "fix" the radiation lesion, and as a suppression of intracellular radio-protecting sulphhydryl concentrations [1,2,18,19].

Besides their radiation sensitizing properties, nitroimidazoles also possess direct cytotoxic effects. One of these cytotoxicities has been found to account for the killing of cells in different systems and appears to be dependent on hypoxia [17,23,26,34,36,37]. Also, well-oxygenized cells can be killed in vivo, however [2,9,28]. This is consistent with the hypothesis that metabolites of misonidazole are produced in the hypoxic tissue regions that are toxic to mammalian cells regardless of oxygenation status.

Radiotherapy has an important role for the treatment of several malignant tumours in the thorax, such as bronchogenic and oesophageal carcinomas. In patients irradiated with a curative intent, regional lymph nodes draining the tumour area are also included in the target radiation volume. Since the trachea is located close to these nodes, this structure (or organ) will almost always receive a high radiation absorbed dose in these patients. If misonidazole is used to augment the therapeutic index of radiotherapy for thoracic tumours, it is therefore important to evaluate the effect of such treatment on the trachea. The model and the methods used in the present study are capable of detecting very early functional and morphological changes in the trachea induced by irradiation and/or drugs.

Materials and methods

The effect of irradiation alone on the trachea of the rabbit has been described in an earlier paper [3]. For the evaluation of the effect of misonidazole, relevant data have been extracted from that work.

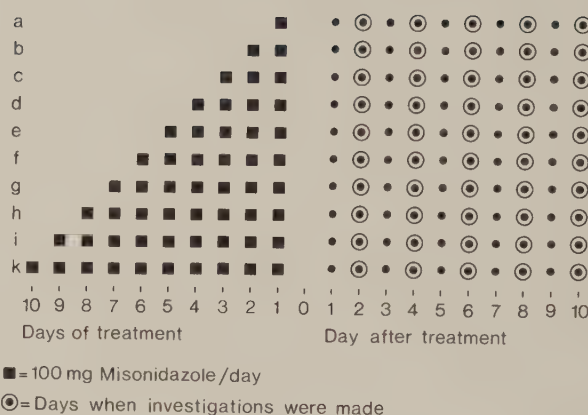


Fig. 1. Treatment schedule for misonidazole. 100 mg was given daily, with a total dose of 100–1000 mg. Experiments were performed each second day after completion of treatment in accordance with the schedule.

The material selected for study consisted of 160 full-grown rabbits weighing 2.0–2.5 kg. They were divided into three groups, each of which was designated a special mode of treatment:

Group I included 10 animals to act as a control group and which accordingly received no treatment at all.

Group II consisted of 50 rabbits which were treated with misonidazole in doses ranging from a total of 100 to 1000 mg according to the treatment schedule shown in Fig. 1. Each one of these animals was

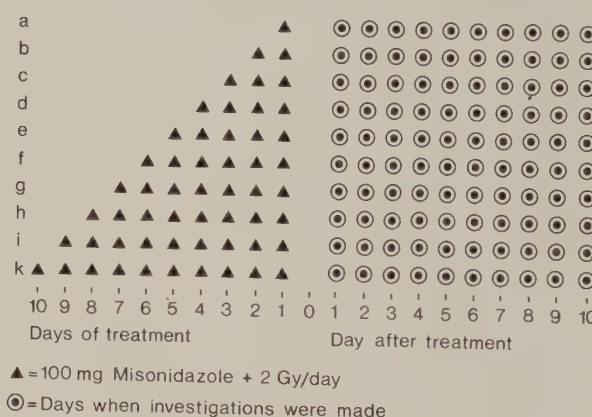


Fig. 2. Treatment schedule for combined misonidazole and irradiation. The drug was given 15–30 min before each irradiation in a dose of 100 mg/2 Gy. The total dose ranged from 100 mg/2 Gy to 1000 mg/20 Gy. After completion of treatment, experiments were made daily from day 1 to day 10.

examined with light microscopy (LM), scanning electron microscopy (SEM), transmission electron microscopy (TEM) and its physiological activity (i.e. its beat frequency) recorded each second day from day 2 to day 10 following the completion of treatment.

Group III was comprised of 100 rabbits which received fractionated irradiation (2 Gy/F) in combination with misonidazole, as shown in Fig. 2. One rabbit was withdrawn from each group daily during 10 consecutive days and was examined with LM, SEM and TEM, together with recording of the ciliary beat frequency.

Experimental procedure

Misonidazole was immediately prior to use dissolved in sterile water and injected intraperitoneally. This was done in the irradiated group 15–30 min before irradiation. The irradiation source was 160 kV X-ray, with HVL: 4 mm Al and SSD: 50 cm. The irradiation target was comprised by the upper part of the ciliated trachea (2 cm).

Ion chambers were used to control the absorbed dose in the trachea. No dose could be measured 15 mm beyond the caudal part of the irradiated area. Each rabbit was anaesthetized about 15 min during the administration of irradiation by intraperitoneal injection of pentobarbital (40 mg per kg body weight).

The first examinations were made 24 h after the completion of treatment. The animal was sacrificed by a blow on the skull and its entire trachea (7 cm in length) was immediately dissected. Samples suitable for LM, SEM and TEM studies were taken at once, both from the apical part of the trachea (T_1) and from its caudal part (T_2) adjacent to the tracheal bifurcation.

Preparations for SEM, TEM and LM were made according to routine methods described earlier [3].

The recording of the mucociliary activity was done using the "indirect reflex method" described by Håkansson and Toremalm [18]. The ciliary beat frequency in the cranial part of the trachea (T_1 , the irradiated area in Group III) was recorded and compared with the beat frequency of the cilia in the

distal part of the trachea (T_2 , the non-irradiated part of the trachea in Group III) in the same animal.

Statistical analysis

The statistical analysis was made using the programme-package MBPD for the analysis of variance and related values. Also regression analysis was used for evaluating the structural changes.

Results

Control Group (Group I)

Ciliary beat frequency

The beat frequency varied between 800 and 1000 beats per minute and in the same animal the quotient between the values from the upper and lower parts was 1.

Light microscopy

LM-observations showed ciliary cells regularly arranged with goblet cells interspaced between them. The height of the ciliary cell layer was $42 \pm 10 \mu\text{m}$, measured from the matrix of the cell to the apex membrane. The height of the ciliary cell layer in the upper and lower parts of the trachea was the same. As to the goblet cells, the normal ratio between them and the ciliated cells was 1/10. It was the same in the upper and lower parts of the trachea.

Ultrastructural findings

SEM of the control preparations showed gracile cilia regularly arranged. A minimum of blebs (0–15) was found on a defined area. The microvilli were intact and no mucous or particles were found covering the ciliary surface (Fig. 8).

TEM of the normal ciliary epithelium showed a layer of basal cells resting on the basal lamina and an apical cell layer comprising ciliated cells, goblet cells and intermediate cells (Fig. 3) [11]. A large, usually lobulated nucleus was found in the basal cells, as well as a sparsity of ribosomes, both rough and smooth endoplasmic reticulum, and a few mitochondria.

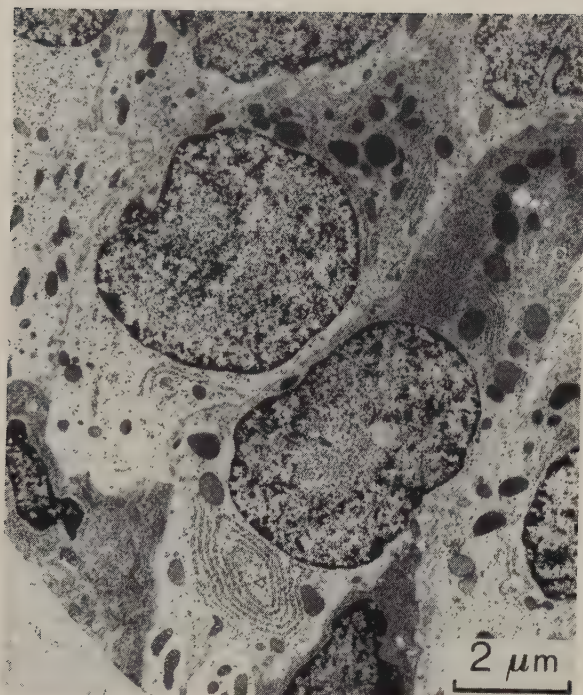


Fig. 3. TEM-micrograph of normal tracheal epithelium. A layer of basal cells resting on the basal lamina and an apical cell layer comprising ciliated cells, goblet cells and intermediate cells can be seen.

The goblet cells reached the apical cell surface, and microvilli were found located at the apex membrane. They did not contain many mitochondria, but were relatively rich in rough endoplasmic reticulum and Golgi complex. Free ribosomes were scattered throughout the cytoplasm. Both small and large numbers of mucous granulae were seen, depending on the condition of the individual goblet cells, and they contained heterogenous material, were of varying size and shape, and surrounded by a membrane.

The ciliated cells had a central nucleus, normally spherical. Mitochondria were scattered throughout the whole ciliated cell, but tended to accumulate at the apex. Adjacent to the nucleus, rough endoplasmic reticulum was seen, and free ribosomes were dispersed throughout the cytoplasm. Golgi complex could be clearly identified in the cells. A rich amount of cilia was located on the surface in the classical 9 + 2 pattern. The cilia were anchored to the cytoplasm by basal bodies.

Misonidazole treatment (Group II)

Fifty rabbits were given misonidazole i.p. with a total dose of 100–1000 mg, according to the treatment schedule seen in Fig. 1.

The physiological and morphological examinations were carried out in exactly the same manner as in Group I.

Beat frequency

The beat frequency in the upper trachea varied between 882 and 1200 beats per minute, and in the lower part it was between 876 and 1238 beats per minute. The quotient between the upper and lower parts in the same animal was 1.

Light microscopy

LM of the preparations showed the same regular pattern found in the control group as to ciliated cells and goblet cells. The ratio of ciliated cells to goblet cells was 10/1. The height of the ciliated cell layer was found to be $43 \pm 10 \mu\text{m}$ (Fig. 4), mea-

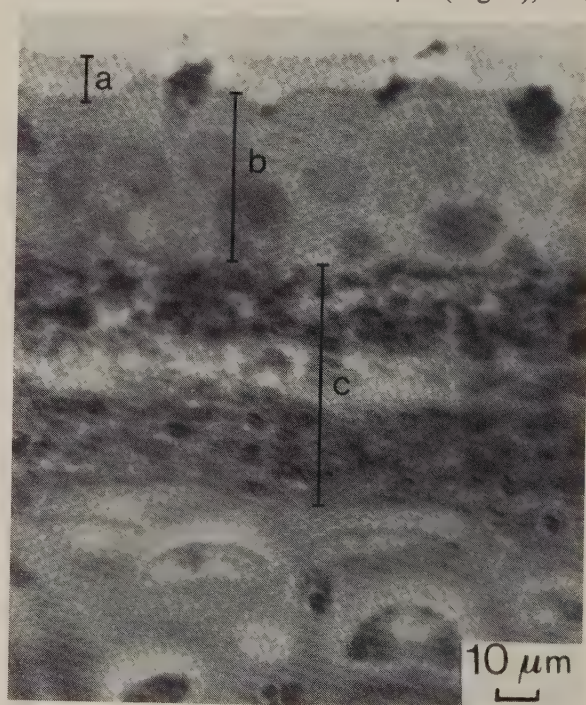


Fig. 4. LM-micrograph of the ciliary epithelium treated with misonidazole. An oedema was observed. a = cilia; b = ciliated cell; c = subepithelial area.

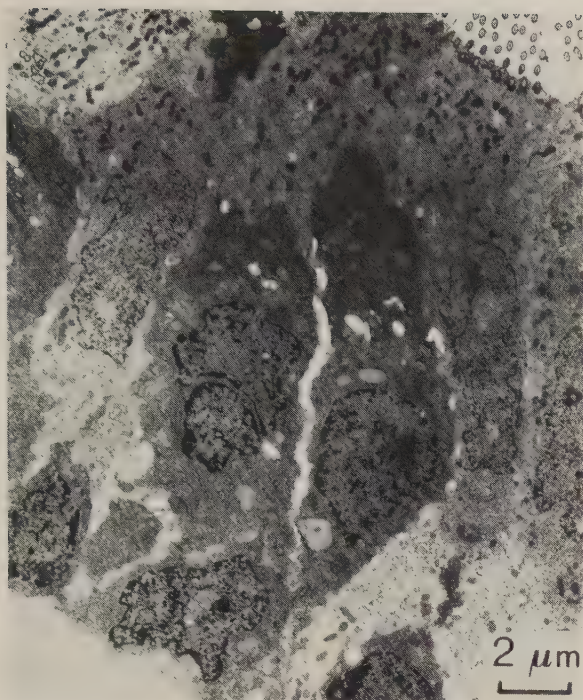


Fig. 5. TEM-micrograph of a rabbit trachea treated with 300 mg misonidazole. Note the hypoplasia with the lack of intermediate cells.

sured from the matrix to the upper part of the ciliary cell membrane. Compared to the control group, the height of the ciliary cell layer was similar, although an hypoplasia was visualized with TEM (Fig. 5). This seemed to depend on an oedema, found in the ciliary cell layer in both the proximal and distal parts of the trachea, and which could also be seen in the sub-epithelial area (Fig. 4). The ratio between the height of the ciliary cell layer in the upper and lower parts of the trachea within the same animal was 1.

Ultrastructural findings

SEM of the trachea in those rabbits treated with misonidazole showed no structural changes compared with the control group. Blebs were found in the same amounts as in the control preparations. The cilia were slender and uniformly organized, the microvilli were scathless and no mucous coating was seen on the surface.

The main finding of TEM was a *hypoplasia* of the

ciliary cell layer. This was first noticeable after 300 mg misonidazole (Fig. 5; Table III) and subsequently recorded throughout the series.

Treatment with combined irradiation and misonidazole (Group III)

Group III was treated with misonidazole in combination with irradiation according to the treatment schedule (Fig. 2). The same investigations were performed as have been described earlier.

Ciliary beat frequency

Examination was made daily from day 1 to day 10 following the completion of each fractionation series. The beat frequency for the entire material varied within the irradiated area (T_1) from 980 to 1508 beats per minute. This concerns both dose and time. The corresponding values found in the non-irradiated area (T_2) were 745–1302 beats per minute. A quotient was determined for each rabbit, which thus served as its own control, on the basis of the values from the upper part of the trachea in relation to those from the lower part and was plotted versus the total dose given (Fig. 6). Each point

Beat frequency quotient, T_1/T_2

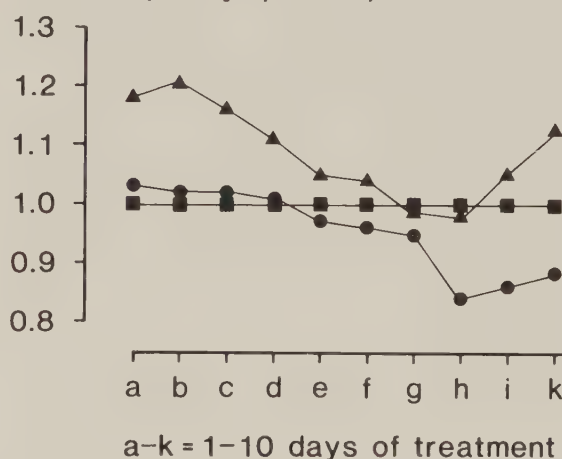


Fig. 6. The ciliary beat frequency within the irradiated part of the trachea (T_1) in relation to the beat frequency of the non-irradiated distal trachea. The regression line for misonidazole and irradiation is $y = -0.006 \cdot x + 1.054$, and for irradiation alone $y = -0.011 \cdot x + 1.049$. ▲, Misonidazole + irradiation; ■, misonidazole; ●, irradiation.

represents the mean value of the quotient for the 10 days following treatment, since statistical analysis showed that there was no significant change in time. This was valid for all of the fractionation groups. Figure 6 showed that those rabbits treated with 100 mg misonidazole and an irradiation dose of 2 Gy showed an increase of the beat frequency up to 16% in the irradiated area. This increase was even more pronounced after 200 mg misonidazole and an irradiation dose of 4 Gy (20%). The curve in Fig. 6 begins to decline at higher doses, but the quotient is still above 1.0 until an administered dose of 14 or 16 Gy combined with misonidazole was absorbed. Further increment of misonidazole and irradiation caused a renewed slight increase of the quotient. After a dose of 300 mg misonidazole and 6 Gy, some spots of the mucous carpet were seen to be inactive, as visualized with India ink.

Statistical analysis of the curve in Fig. 6 revealed a significant dose-dependent decrease ($p < 0.0001$).

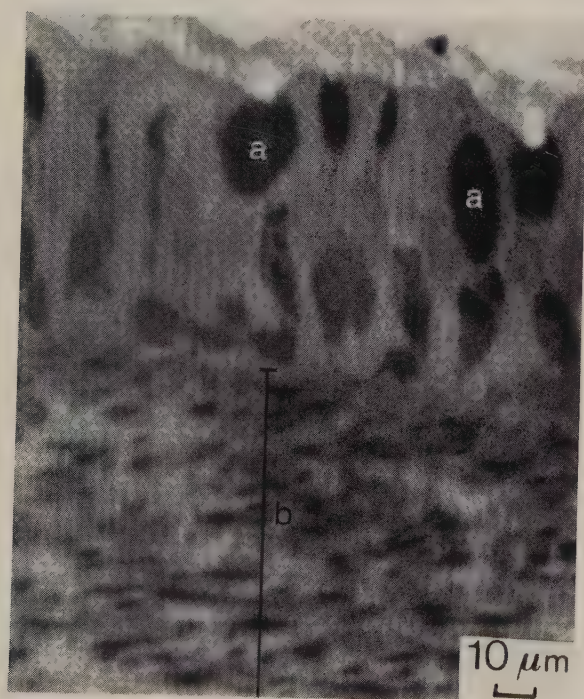


Fig. 7. LM-micrograph of the ciliary epithelium treated with 600 mg misonidazole + 12 Gy, examined 24 h after irradiation. a = goblet cells; b = subepithelial layer. Note the oedema of the epithelium, the rich amount of goblet cells and the hyperplasia.

TABLE I

The relationship between the height of the ciliary epithelium in the normal trachea, in the trachea treated with misonidazole and with combined misonidazole and irradiative treatment.

	μm	Interrelation
Control	42 ± 10	1.0
Irradiation ^a	64 ± 20	1.5
Misonidazole	43 ± 10	1.0
Misonidazole + irradiation	82 ± 20	2.0

^a Data extracted from ref. [3].

Light microscopy

LM-micrographs were taken of the upper (T_1) and lower (T_2) parts of the trachea. All of the preparations showed an oedema of the ciliary epithelium (Fig. 7). The height of the ciliary cell layer in the upper part of the trachea was $82 \pm 20 \mu\text{m}$ (Table I), which in comparison with that of the control animals was an increase of 2.0. This enlargement within the irradiated area had the appearance of both a hyperplasia of the ciliary epithelium and of an oedema, which was already noticeable within the low dose range and throughout the entire series. The ratio of ciliated cells to goblet cells, calculated from LM-micrographs, was increased from 10/1 to 10/4 (Fig. 7). It was observed at low doses and was constant throughout the series at all of the administered doses.

Scanning electron microscopy

A scoring system has been drawn up in an attempt to classify the varying degrees of reaction in relation to dose.

Scoring of the SEM-pictures from all of the 100 rabbits was done by three persons. In order to obtain a gliding scale and possibly enable more accurate evaluation, half-values have been included in the scoring system.

The SEM-scoring system

The following definitions were worked out for the respective values used:

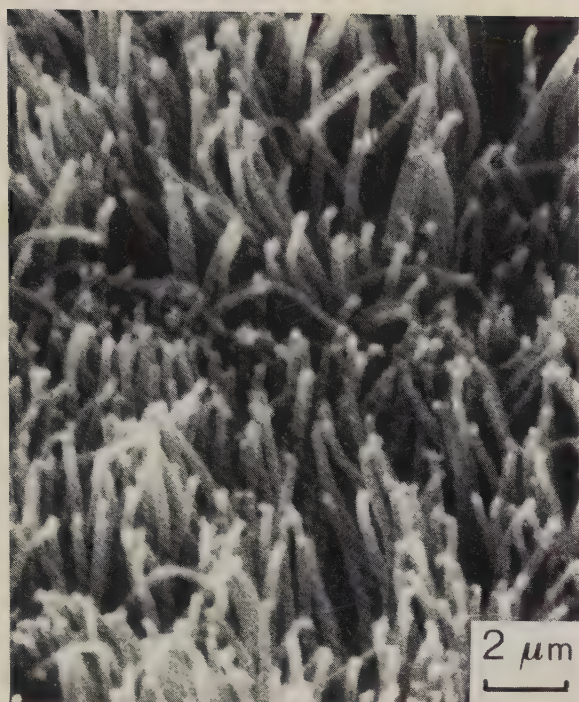


Fig. 8. SEM-micrograph of normal tracheal epithelium. The cilia are gracile, regularly arranged and have a minimum of cytoplasmic (vesicular) extrusions.

0.0: (Fig. 8) Normal surface with clean cilia having a minimum of blebs (≤ 25 on a defined area $\times 5000$ magnification) and with regular arrangement. In rare cases with visible goblet cells having intact microvilli. No mucous granulae or fibrin.

1.0: (Fig. 9) The cilia have a regular pattern, which is constant, as can be seen on a $\times 1000$ picture. Blebs occur especially on the tips of the cilia, but also on the sides (in a number up to 50 on a defined area at $\times 5000$ magnification). Only solitary mucous granulae are to be found on the surface. The goblet cells have normal microvilli.

2.0: (Fig. 10) Blebs in a number of 51–75 on a defined area at $\times 5000$ magnification. A rich amount of goblet cells are seen protruding on the surface with disintegrated and shortened microvilli. Some of the goblet cells have emptied their contents, and mucous granulae are seen on the surface. Many cilia are seen adhering to one another.

3.0: (Fig. 11) Blebs occur in a number of 76–100 on a defined area at $\times 5000$ magnification. The cilia



Fig. 9. Score 1.0. SEM-micrograph of the cilia treated with 100 mg misonidazole + 2 Gy. The cilia are regularly arranged. Increased numbers of blebs are to be seen, especially on the tips of the cilia. Arrow = goblet cell with microvilli.



Fig. 10. Score 2.0. 400 mg misonidazole + 8 Gy. Note the increased amount of blebs (arrows).



Fig. 11. Score 3.0. 800 mg misonidazole + 16 Gy. There is a rich amount of blebs. The cilia are clustered together, and in some areas have a fine fibrillar network between them.

are arranged in a regular pattern. Thick masses of emptied goblet cells with mucous granulae are spread over the surface; the remaining goblet cells have almost no microvilli. There is a coating of fibrin and the cilia are connected by fibrin threads.

4.0: (Fig. 12) The cilia are in a disorderly pattern and have disintegrated. The goblet cells are emptied. The surface is covered with detritus and fibrin threads. A few cilia with intact structure can be seen here and there.

No statistically significant time variations during the 10-day observation period were noted in any of the 10 different groups ($p > 0.1$). It was therefore possible to connect all of the score-values from one single group to one point and plot them in a diagram versus the total dose (Fig. 13). Each point represents the mean value for the three persons doing the scoring. Statistical analysis of these results shows a significant effect of the given dose versus the structural changes observed ($p < 0.01$).

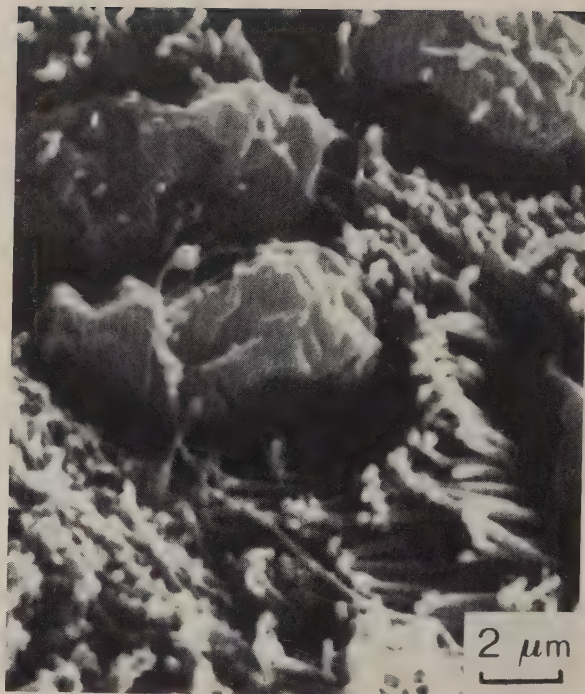


Fig. 12. Score 4.0. 100 mg misonidazole + 20 Gy. Emptied goblet cells with no microvilli. There is detritus on the surface. The cilia are disarranged and disintegrated.

Transmission electron microscopy

TEM-observations revealed a remarkable increase in the number of mitochondria at the apex of the

Average reaction

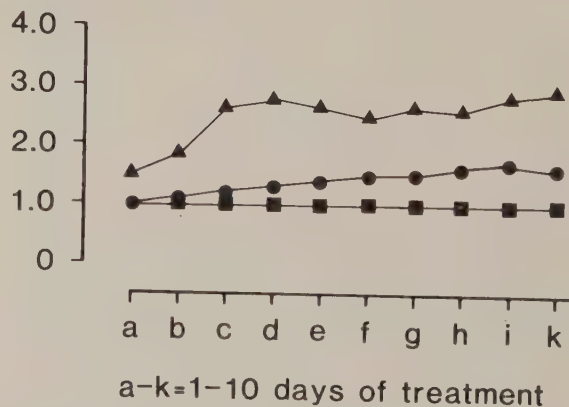


Fig. 13. Mean value of the scores of the SEM-micrographs, as judged by three persons. ▲, Misonidazole + irradiation; ■, misonidazole; ●, irradiation.

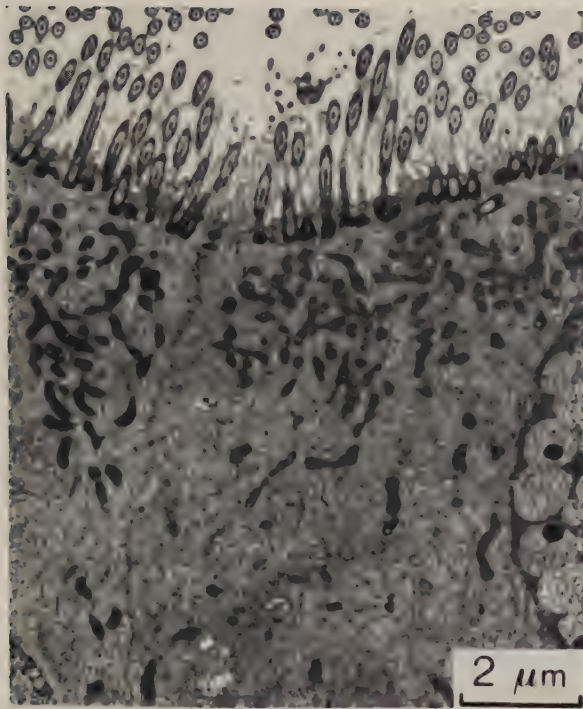


Fig. 14. TEM-micrograph of the trachea of the rabbit treated with 100 mg misonidazole + 2 Gy. Note the increased amount of mitochondria apically in the cell.

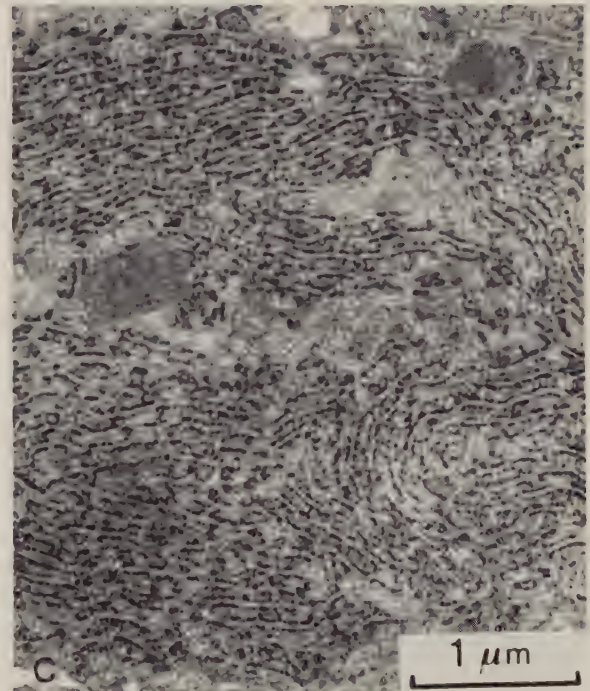
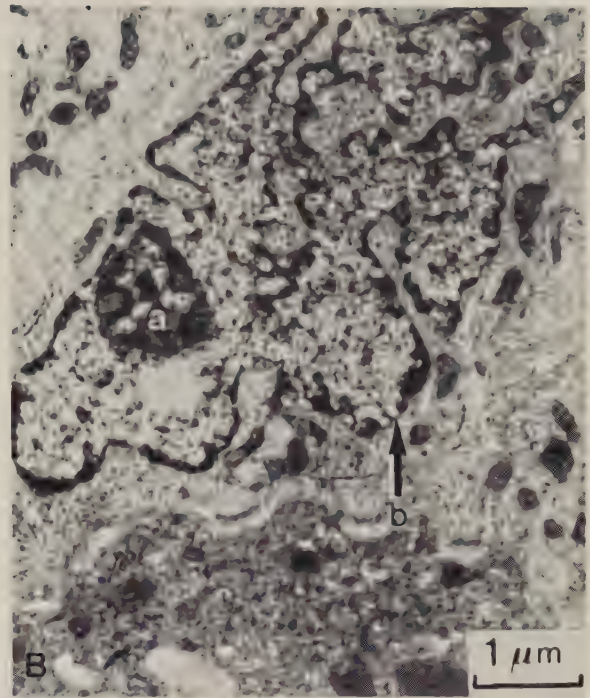


Fig. 15(A) TEM-micrograph of the trachea of the rabbit after treatment with 900 mg misonidazole + 18 Gy. Rich amount of goblet cells (arrow). (B) Multilobulated nucleus with marked nucleoli (a), increased numbers of nuclear pores (b). (C) Endoplasmic reticulum with ribosomes.

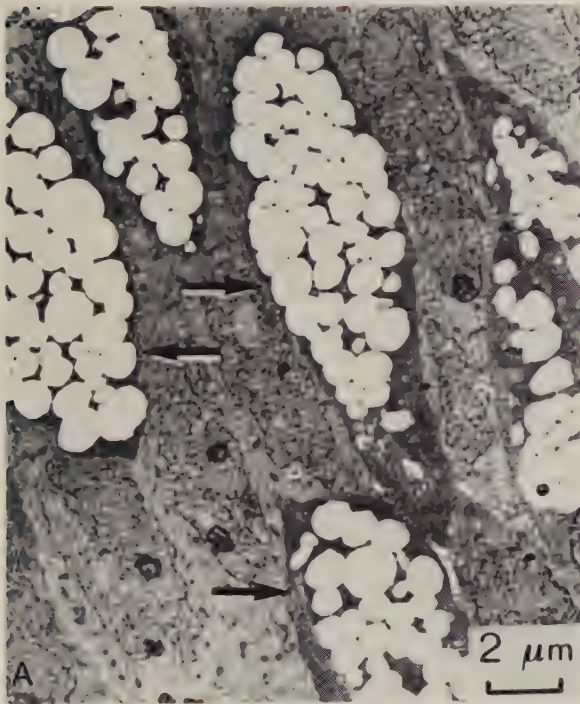


TABLE II

Estimation of the number of mitochondria apically in the ciliated cell on a determined area.

Days of treatment (see Fig. 2)	Controls	Irradiation ^a	Misonidazole	Misonidazole + irradiation
a,b,c,d	+	++	+	+++
e,f,g	+	+	+	++
h,i,k	+	+	+	+++

^a Data extracted from ref. [3].

+ = ≤29; ++ = 30-59; +++ = ≥60.

cells (Fig. 14), seen from the beginning of the series up to the irradiation dose of 10 Gy. At misonidazole administered with 12 or 14 Gy irradiation, this phenomenon was not seen to the same extent as at lower doses, but it occurred again at higher doses (Table II). The cells seemed to be very active, having multilobulated nuclei, increased numbers of nuclear pores, marked nucleoles, a rich amount of rough endoplasmic reticulum and free ribosomes (Fig. 15 a-c). A hyperplasia with 5 or 6 cell layers was observed in doses of misonidazole combined with 8 Gy irradiation and higher doses throughout the series (Table III). This seemed to be due to an increased number of intermediate cells. The goblet cells looked swollen, contained a rich amount of polymorphic granulae and varied in shape according to their physiological condition, although they were increased in number (Fig. 15a). Cell damage

and cell-death with the loss of cytoplasmic organelles were predominantly seen in the intermediate cell-population, and could be seen at random throughout the entire series.

Discussion

Physiology

In vitro investigations [5] of the ciliary epithelium during exposure to radiation have shown a stimulation with an increase of the ciliary beat frequency around 15-20%, lasting for 10 sec, whereafter it returned to its normal value. This stimulating effect has also been observed when the trachea of the rabbit was irradiated in vivo [3]. The phenomenon occurred at doses as low as 2 and 4 Gy during the first

TABLE III

The number of cell layers visualized on a TEM-micrograph.

Days of treatment (see Fig. 2)	Controls	Irradiation ^a	Misonidazole	Misonidazole + irradiation
a,b,c	++	++	++	++
d,e,f	++	++	+	+++
g,h,i,k	++	++	+	+++

^a Data extracted from ref. [3].

+ = 1-2 cell layers (basal cells, apical cells, no intermediate cells); ++ = 3 cell layers (basal cells, apical cells, intermediate cells); +++ = >3 cell layers (basal cells, apical cells, increased number of intermediate cells).

day of observation following irradiation, and was in the range of 5–10%.

The acceleration of beating within seconds *during* irradiation [5] is believed to depend on mechanisms separated from the ones responsible for the increase of the beat frequency seen 1–10 days after irradiation. The rapidity of the immediate response speaks for processes within the cilium itself, perhaps an increased available amount of ATPase, or by changes in the membrane permeability of the cilia, allowing some ionic fluxes which could alter the rate of ciliary beating.

In the normal trachea, the ciliary beat frequency is about 1000 beats per minute. Treatment with misonidazole alone did not seem to cause any changes in this physiological activity. The combination of the drug with irradiation, however, caused a *significant* increment of the beat frequency during the entire 10-day observation period, and was most pronounced within the lower dose range (around 20%, see Fig. 6).

The stimulation of the beat frequency seen in this investigation after treatment with *misonidazole and irradiation* probably depends on effects on the energy-production system within the cell, with an increased amount of ATP released from the mitochondria. The theory that ATP serves as an energy for ciliary motility and that the ATP-concentration determines the beat frequency has been suggested as early as 1974 [30]. The finding in the present investigation that the total amount of mitochondria within the apical part of the ciliated cell increased 2 to 3 times (Table II; Fig. 14) supports the hypothesis that the increased ciliary activity is related to a stimulation of processes within the cell.

Morphology

LM-micrographs of the preparations treated with misonidazole showed that the height of the epithelium was unchanged, although a hypoplasia was noticeable on TEM-preparations. The reason for this was found to be an intracellular swelling. In the subepithelial area, an interstitial oedema was detected, the etiology of which is probably multifactorial. In a recent study (Albertsson et al., to be

published) on the effect of misonidazole $\pm$ irradiation on the microcirculation of the trachea, it could be seen that the drug alone was able to increase the vascularity by a factor of 3 in the subepithelial cell layer. The same result was obtained when the drug was combined with irradiation. Since the number of capillaries stayed unchanged, this phenomenon has been interpreted as being due to capillary dilatation. Such a dilatation is known to result in an increase in capillary blood pressure favouring an increased filtration over the capillary membrane, resulting in increased flow into the extracellular space [24]. The intracellular swelling is therefore probably secondary, dependent on an intensified flow of fluid into the cells by diffusion and osmosis. In the group treated with the drug and irradiation, radiation damage on the membranes may facilitate and perhaps accelerate the fluid-flow by mechanisms described above. This group also had an enlargement of the ciliary epithelium by a factor of 2, but this heightening was not a pure oedema, since a hyperplasia was also observed within this group (Table III).

The stimulatory events also included the goblet cells, which were increased in number 3 times in the group treated with misonidazole combined with fractionated irradiation (Fig. 4). The drug itself did not cause any change in the number of goblet cells. The stimulating effect of irradiation on goblet cells has been described earlier, both in the intestine [16], and in tracheal epithelium [3]. TEM-observations showed a hypoplasia of the ciliary epithelium in the group treated with misonidazole alone (Table III; Fig. 5), mainly seen as a lack of the intermediate cell layer. This might be due to a direct toxic effect of the drug on this tissue. The only organ-specific toxicity thus far described for misonidazole is on the nervous system, giving convulsions and EEG-changes in the human, and causing sensory neuropathy following axonal degeneration and demyelination of the peripheral nervous system in the human and the mouse [10,29]. Even if cytotoxicity of misonidazole, measured as cell-killing, is a consequence of reductive metabolism of the drug in which a cytotoxin is produced, and therefore has hypoxia as a prerequisite, misonidazole can also

affect well-oxygenated cells. With the use of *in vivo* or spheroid systems, it has been proposed that metabolites of misonidazole can leak over from hypoxic to oxic regions, where they exert cytotoxic effects [31]. Deys et al. [14], however, found a prolongation of cell-cycle time, abnormal mitoses and impaired cloning capacity for well-oxygenated mouse osteosarcoma cells, also *in vitro*, and Lindmo et al. found a lengthening of cell-cycle time, affecting all phases in human NKIH cells, cultivated *in vitro* [22]. The assumption that all normal tissues are well-oxygenated can also be questioned, from the analysis of results from a variety of rodent tissues which showed radiosensitization with irradiation and misonidazole (for an overview, see Stewart et al. [32]). Whether the trachea is such a tissue, containing a certain proportion of hypoxic cells, does not seem to have been investigated. Anaesthetics are known to reduce the radiosensitivity of many tumours [13,25,28], and some normal tissues as well [4,21]. In a recent study, pentobarbitone gave significant radioprotection to the lung, with breathing frequency as the functional endpoint, but this could be removed by the addition of misonidazole [15]. Misonidazole did not sensitize the lungs of unanaesthetized mice to the effects of irradiation in that study. Radiosensitization by misonidazole has also been reported for the spinal cord of anaesthetized rats [38], but not for unanaesthetized mice [35]. It therefore seems that the evaluation of normal tissue-effects of misonidazole with or without irradiation can be highly influenced by anaesthesia. Since pentobarbital was used for anaesthesia in the present investigation, the above-mentioned data should be borne in mind when interpreting the results.

SEM-investigations of preparations from rabbits treated with misonidazole showed no structural changes in comparison with the control group. The combination of the drug with irradiation seems to cause increasing degrees of damage with increasing dose. The phenomena observed were blebs, clustering of the cilia, connection between the cilia through a fibrillar network, empty shells from goblet cells and mucus. At the highest doses, the microvilli were lost and the goblet cells showed a

smooth surface. The signs of damage were clearly dose-dependent, increasing with increasing dose (Figs. 9–13).

The combination of the drug and irradiation caused quite different phenomena in the TEM-preparations. Cells seemed very active, with multilobulated nuclei, increased numbers of nuclear pores, marked nucleoles, a rich amount of endoplasmic reticulum and free ribosomes.

All these observations together indicate an increased metabolic activity of the ciliated cells. This was also reflected in the increased physiological activity observed in this group, and in the hyperplasia of the epithelium. The combination of misonidazole and irradiation seems to have stimulatory effects on the ciliary epithelium which are in contrast to the toxic effects caused by the drug when given alone. The basis of these stimulatory mechanisms is not fully understood.

Conclusion

Fractionated irradiation of the ciliary epithelium in the trachea of the rabbit has shown dose-dependent physiological and morphological effects. Misonidazole potentiates these effects of radiation with a more pronounced change of the ciliary beat frequency and an increased metabolic activity as could be visualized on TEM. The combination of drug and irradiation also induced a hyperplasia of the ciliary epithelium. Misonidazole itself has no effect on the ciliary beat frequency, but causes a hypoplasia of the ciliary epithelium.

Acknowledgements

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Reaction of the vascular system in the trachea of the rabbit exposed to fractionated irradiation with and without the addition of misonidazole

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Summary

As the radiation field used in the radiation therapy of malignancies in the thoracic cavity often exposes the trachea to ionizing irradiation, it is important to ascertain the effects of radiation on this tissue either as a single therapy or in combination with radiosensitizers. In the study reported here the vascular area in the subepithelial layer of the trachea has been calculated in 160 rabbits treated in four ways: (1) 10 rabbits received no treatment and served as controls; (2) 50 rabbits were given 100 mg misonidazole daily on consecutive days, with the individual total dose ranging from 100 to 1000 mg; (3) 50 rabbits were treated with misonidazole in the same way, but were also exposed to radiation (2 Gy/F) at 15–30 min later; (4) 50 rabbits received only fractionated radiation (2 Gy/F). The total radiation dose in the irradiated animals ranged from 2 to 20 Gy. In the treated groups, an oedema was observed in both the ciliary cell layer and in the subepithelial area. In the group given only irradiation, this oedema was dose-dependent, but no such dose-dependency was observed in the two groups treated with misonidazole. The vascular area in the groups treated with misonidazole was significantly increased as compared with the group given only irradiation and the control group; this was valid both with and without correction for the oedema. There was a significant correlation between the oedema and the vascular area in the groups treated with misonidazole, which was not found in the group irradiated without the drug.

Introduction

Modern oncology has devoted much time and effort to the search for new drugs capable of augmenting the effects of the irradiation treatment of tumours – drugs which are classified as radiosensitizers.

An investigation of a group of chemical agents has recently been conducted with special attention to the nitroimidazoles, belonging as they do to substances with a high electronic affinity and in which radiosensitization has a direct relationship to the redox-potential [2]. These compounds, moreover, seem to act by decreasing the intracellular concen-

trations of thiols [13]. They increase the radiosensitivity of hypoxic cells to almost the same level as that of oxygenated cells [1-3].

Two substances have proved to be of special clinical interest: metronidazole [2] and misonidazole [10,11]. Misonidazole is the most effective of these two, but also the most toxic, having harmful effects, especially on the peripheral nerves [21,27]. Its action is also strictly dose-dependent [21]. The therapeutic value of this drug, which *in vitro* has an oxygen enhancement ratio of up to 2.0 [3,12], is restricted by its neurotoxicity. Misonidazole also has a cytotoxic effect on hypoxic cells which diverges from its radiosensitization mechanism [24,25]. Radiochemical determinations of its sensitizing effect [4] show that this occurs very rapidly, giving rise to the question of whether misonidazole is supplied to the tissues with oxygen by active transportation or by some action on the vascular system or whether this is due to mediation of the radiosensitizing effect by some other process or combination of events. These processes are interdependent, but the radiosensitizing action of misonidazole occurs much more rapidly than its cytotoxic effect. It has been found that misonidazole enhances the effects of irradiation on certain normal tissues. Studies with hyperbaric oxygen and misonidazole have shown that some normal tissues contain a significant number of hypoxic cells and this should be considered when using hypoxic cell sensitizers [23]. It is not known whether or not the trachea is such a tissue and therefore should be regarded as radiobiologically hypoxic. We have therefore investigated the action of misonidazole on the subepithelial layer of the trachea of the rabbit and the changes seen after drug administration in combination with irradiation. The experimental system used was well-suited for detecting changes in the vascularity. An attempt was made to closely simulate the doses of misonidazole and irradiation used in routine clinical practice. The fractionation time parameter however, was kept constant.

Materials and methods

A total of 160 rabbits with body weights varying between 1.8 and 2.3 kg and which were divided into four groups given different modes of treatment was used. Ten rabbits received no treatment at all and served as controls. Fifty were subdivided into groups of five and given fractionated irradiation (2 Gy/F), the total dose varying from 2 to 20 Gy. The radiation was given daily on consecutive days according to the schedule shown in Fig. 1. One animal was normally taken out of the group every second day after the completion of irradiation and examined. Fifty rabbits were treated with misonidazole daily on consecutive days with a total dose ranging from 100 to 1000 mg (Fig. 2). The drug was suspended in sterile water and injected intraperitoneally. One animal in each of the subgroups was examined every second day from day 2 to day 10. Another 50 rabbits were treated in groups of five with fractionated irradiation (2 Gy/F) strictly following the fractionation schedule described earlier, but they also received 100 mg misonidazole given intraperitoneally at 15-30 min prior to irradiation.

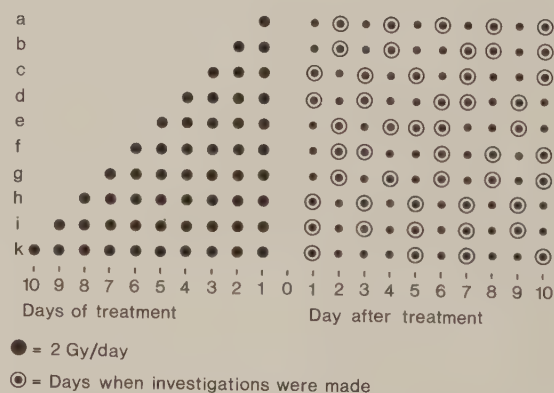


Fig. 1. Fractionated irradiation schedule: 2 Gy/F was given daily, with the total dose ranging from 2 to 20 Gy. Examinations were normally made every second day after the completion of irradiation, from day 2 to day 10 (days marked with circles).

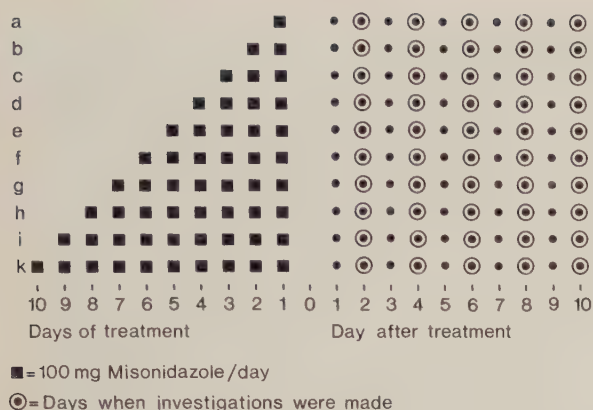


Fig. 2. Misonidazole was given daily on consecutive days, with the total dose ranging from 100 to 1000 mg. Examinations were made every second day from day 2 to day 10 (days marked with circles).

One animal from each subgroup was normally examined each second day after completion of irradiation (Fig. 3). Only rabbits subjected to irradiation were given pentobarbital anaesthesia.

Irradiation The irradiation source was a 160 kV X-ray apparatus; HVL: 4 mm Al; SSD: 50 cm; dose rate = 0.013 Gy/s. The irradiated segment of the trachea was 20 mm of the cranial part. The absorbed dose in the trachea was controlled by ion chambers and thermoluminescence dosimeters. Each rabbit was anaesthetized with pentobarbital

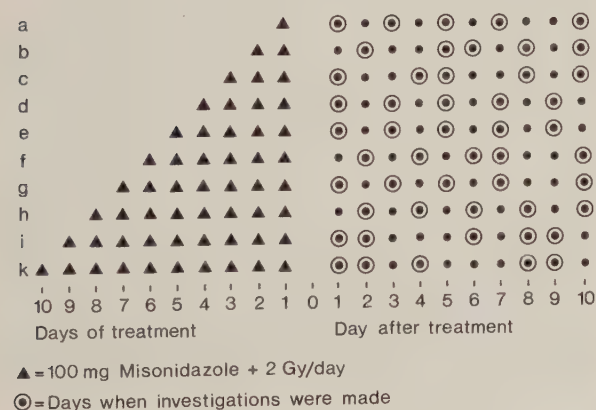


Fig. 3. Fractionation pattern when misonidazole was also given at 15-30 min prior to each irradiation. Examination days are marked with circles.

(40 mg/kg body weight), given intraperitoneally prior to each irradiation, during which the animal was laid on its back with its extremities stretched out.

Preparation for light microscopy (LM)

Specimens were taken from both the upper and lower parts of the trachea in each rabbit and examined by LM. The preparations were originally done for electron microscopy. In the present experiment, the LM was performed on 1 μ m sections. The specimens were placed in 2.5% glutaraldehyde in 0.15 M cacodylate buffer (pH = 7.3) for 12 h and then transferred into the same buffer; they were later fixed in 1% osmium tetroxide in 0.15 M cacodylate buffer for 2 h. The specimens were dehydrated in ethanol and embedded in EPON. One- μ m-thick sections were cut, stained in methylene blue and examined in a Zeiss light microscope.



Fig. 4. Light microscopy of the nonirradiated trachea in a control animal. a = Ciliary cell layer; b = subepithelial area; c = subepithelial lamina; d = blood vessel; e = cartilage.



Fig. 5. Light microscopy of irradiated trachea (16 Gy; 2 Gy/F; 8 days after treatment). Note the oedema of the ciliary epithelium and the subepithelial area. a = Ciliary cell layer; b = subepithelial area; c = subepithelial lamina; d = blood vessel; e = cartilage.

Method for estimation of the vascularity

Each preparation was examined by LM and three consecutive micrographs covering the epithelial and the subepithelial areas were taken. Figures 4–7 represent LM micrographs, one from each group, and Fig. 8 represents a schematic drawing of the area. The height of the ciliary epithelium was measured, as was that of the layer of collagen fibres just beneath the basal membrane – the so-called subepithelial lamina. Then, the subepithelial area was measured, i.e. the area between the ciliary epithelium and the cartilage arcs. All of the blood vessels (arteries, veins, capillaries) within the area were then identified and marked with a line on the inner side of the endothelium. The area of the blood vessels was calculated in the following way. The broadest diameter of the vessel was identified and multi-

plied by its longest perpendicular diameter. The entire blood vessel area was then related to the total subepithelial area as a quotient, the subepithelial area being the denominator. Each vessel was approximated for a quadratic area, since the area shapes differed greatly, ranging from circular to very thin elliptical forms. The largest error will then be the deviation of the area from the quadrate to the circle and will differ maximally by a factor of 1.27. The error becomes less, the more elliptical the vascular area is. Since most of the vessels were elliptical, this error is considered as small.

Statistical analysis

The statistical analysis of the material was performed by analysis of variance and related methods. Regression analysis and normal tests were also used.

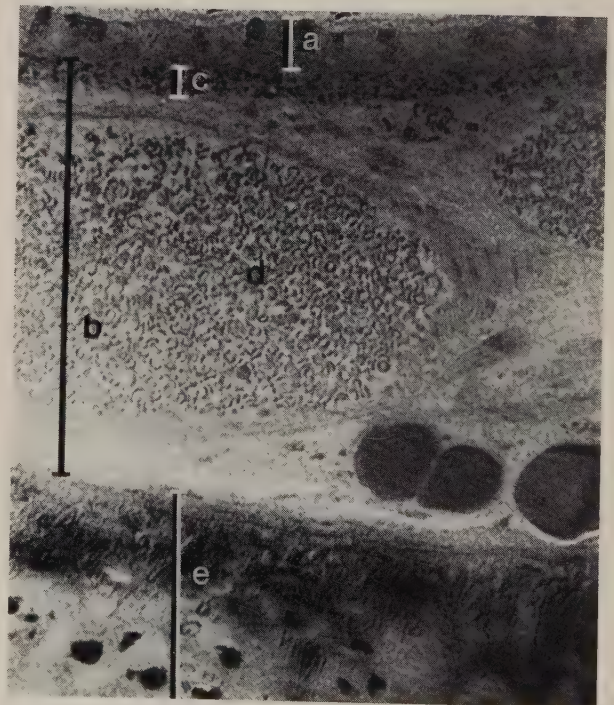


Fig. 6. Light microscopy of the trachea treated with misonidazole. Note the oedema and the increased vascularity. a = Ciliary cell layer; b = subepithelial area; c = subepithelial lamina; d = blood vessel; e = cartilage.



Fig. 7. Light microscopy of the trachea treated with fractionated irradiation plus misonidazole. Note the increased vascularity and dilatation of the blood vessels. a = Ciliary cell layer; b = subepithelial area; c = subepithelial lamina; d = blood vessel; e = cartilage.

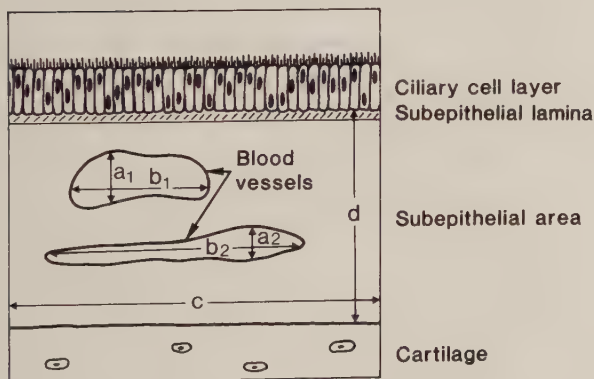


Fig. 8. Schematic drawing showing the basis for estimation of the vascularity within a selected subepithelial area. Vascular area: $\frac{(a_1 \times b_1) + (a_2 \times b_2)}{c \times d}$

Temperature measurements

In all of the 160 animals, the temperature was recorded with a thermistor beneath the *pars membranacea* prior to the experiment where radiation was administered and this temperature was then compared with that of the corresponding nonirradiated part at the distal part of the trachea.

Results

Oedema reference material

Determination of the extent to which interstitial swelling was induced in the experiments was done by using two parameters thought to be representative: (1) the height of the ciliary cell layer; and (2) the height of the subepithelial lamina. The latter was used because it was very difficult to make a more exact measurement of the whole subepithelial area. Two types of controls were used. First, the 10 untreated rabbits were investigated (Control a). The data from this group were compared with the values for the lower nonirradiated part of the trachea taken from the 50 irradiated rabbits in which the upper part of the trachea was irradiated (Control b). In all cases, each of the animals had that part of the trachea as its own control. The purpose of this comparison was to determine if there was any "diffusion flare" from the irradiation.

The height of the *ciliary layers* in these two con-

TABLE I

Height of the ciliary cell layer as measured in the control animals (a), in the lower control part of the trachea in the animals treated with fractionated irradiation (b) and in the animals treated with misonidazole, irradiation or a combination of the two.

	μm	Interrelation
Control a	42 ± 10	1.0
Control b	43 ± 10	1.0
Irradiation	64 ± 20	1.5
Misonidazole	43 ± 10	1.0
Misonidazole + irradiation	82 ± 20	2.0

TABLE II

The height of the subepithelial lamina measured in the control animals (a), in the lower untreated part of the trachea in the animals treated with fractionated irradiation (b) and in the animals treated with misonidazole, irradiation or a combination of the two.

	μm	Interrelation
Control a	26 ± 5	1.0
Control b	30 ± 12	1.1
Irradiation	54 ± 14	1.9
Misonidazole	40 ± 10	1.4
Misonidazole + irradiation	62 ± 20	2.2

tol groups is shown in Table I. Values differ from $42 \pm 10 \mu\text{m}$ in the 10 nonirradiated rabbits (Control a) to $43 \pm 10 \mu\text{m}$ in the 50 animals where the upper part of the trachea was irradiated. The difference between these two values is apparently not significant, so that the value for the latter group can therefore be used as a reference value (1.0) for further comparisons.

The results of the measurements of the height of the *subepithelial layers* are shown in Table II. The value for Control a was $26.5 \pm 5 \mu\text{m}$, but for Control b it was $30 \pm 12 \mu\text{m}$. This difference is also not statistically significant and the average is therefore used as a reference value.

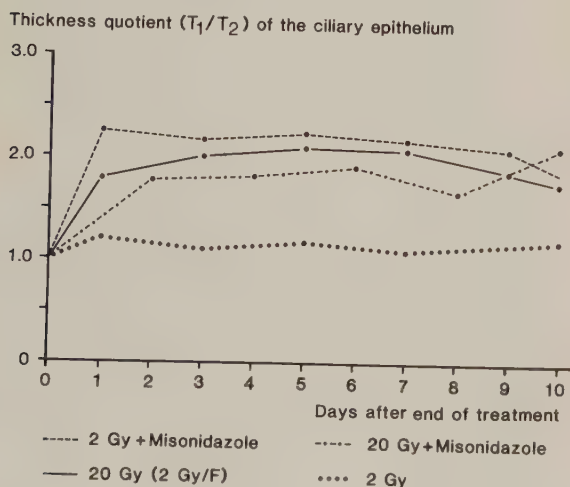


Fig. 9. The relative thickness of the ciliated cell layer compared with the controls. T_1 = irradiated trachea; T_2 = nonirradiated trachea 4 cm distal to the irradiation field.

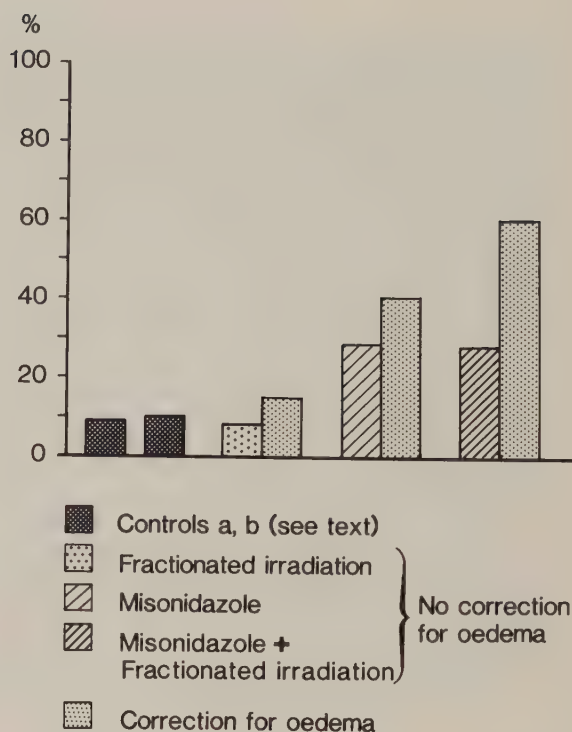


Fig. 10. The mean of the vascular area as measured in percent of the area of the subepithelial layer, with and without correction for the oedema.

Subepithelial vascularization: reference material

In the 10 untreated rabbits (Control a), the area of vascularization was estimated to be 9% and in the 50 where measurements were made from the lower part of the trachea, where the upper part had been irradiated, the area of vascularization within the lower part was 10% (Fig. 10). This represented no statistical difference. Since the latter group of rabbits were given pentobarbital anaesthesia before the irradiation, this finding suggests that anaesthesia in itself had no effect on vascularity in this respect.

Oedema: irradiated material

In the irradiated rabbits ($n = 50$), the height of the ciliary cell layer was $64 \pm 20 \mu\text{m}$, and in comparison with the controls this represented an increase by a factor of 1.5 (Table I). This increase was statistically significant ($p < 0.0001$). The oedema of

the irradiated rabbits was dose-dependent. It increased with increasing dose and, after 20 Gy (2 Gy/F), the irradiated epithelium (T_1) was twice as thick as the cell layer 4 cm from the irradiated field (T_2) (Fig. 9). The height of the subepithelial lamina was $54 \pm 14 \mu\text{m}$ (Table II). This was an increase by a factor of 1.9 in comparison with the controls. This was also statistically significant ($p < 0.0001$).

Subepithelial vascularization: irradiated material

Without correction for oedema

In the 50 irradiated rabbits, the vascularity was 8%. There was no statistically significant difference between the irradiated group and the control groups in this respect ($p = 0.16$). No significant change with increasing dose was found ($p = 0.34$), nor was there any effect of time after treatment ($p = 0.55$). All of the values from the irradiated group could therefore be pooled (Fig. 10).

With correction for oedema

In the animals treated with fractionated irradiation, although the relative percentage of the vascular area was 8%, it increased to 15% when corrected for the oedema on the basis of the swelling of the subepithelial area (oedema factor = 1.9). If the percentual swelling of the ciliated cells is used (oedema factor = 1.5), the increase is at least 12%. These values show a statistically significant increase of the vascular area as compared with the control groups ($p < 0.01$).

Oedema: misonidazole-treated animals

Measurement of the height of the ciliary cell layers in the group of 50 rabbits given misonidazole showed a value of $43 \pm 10 \mu\text{m}$. This was not statistically different from that of the control groups. The subepithelial lamina, however, had a statistically significant ($p < 0.001$) heightening, with a value of $40 \pm 10 \mu\text{m}$. This heightening was established within the low dose range and maintained throughout the series with no dose-dependency. These val-

ues appeared to indicate a discrepancy between the oedema factors. In the former case, this height was the same as in the controls. The cause of this has been studied earlier and is discussed in another report [6].

Subepithelial vascularization: misonidazole treatment

Without correction for oedema

Determinations of the vascularity in the groups of 50 rabbits given misonidazole showed a drastic increase, with an area of vascularization amounting to 29%. There was a tendency to decreasing values with increasing time after completion of irradiation, although this was not statistically significant. No increasing effect with increasing dose was found. All of the values could therefore be connected to one point (Fig. 10). The subepithelial vascular areas in the groups treated with fractionated irradiation and in the control groups were significantly smaller than in the group treated with misonidazole ($p < 0.0001$).

With correction for oedema

When the oedema factor 1.4 (Table II) was used, misonidazole led to a significant enlargement of the vascular area: 40% ($p < 0.00001$) as compared with the irradiated group and the control groups (Fig. 10). For certain reasons (vide Discussion), the factor 1.0 from Table I could not be applied.

Oedema: misonidazole plus irradiation

In the group of 50 rabbits treated with misonidazole plus irradiation, a significant oedema ($p < 0.0001$) of the ciliary epithelium was found ($82 \pm 20 \mu\text{m}$, Table I) and of the subepithelial lamina ($62 \pm 20 \mu\text{m}$, Table II). This phenomenon was seen during the first days of study in the low dose range and was maintained within small variations during the 10 days following treatment, with no significant dose-dependency (Fig. 9).

Subepithelial vascularization: misonidazole plus irradiation treatment

Without correction for oedema

Within this group, measurements of the vascular area showed a value of 28%. Neither a significant dose or time effect could be established. All of the values could therefore be connected to one point (Fig. 10). This value of the vascular area was significantly increased as compared with the irradiated group and the control groups ($p < 0.0001$), but not with the group treated with misonidazole alone.

With correction for oedema

With a correction factor of 2.2 (Table II), the vascular area was 61% and with a correction factor of 2.0 (Table I) it was 56%. This was a statistically significant increase as compared with the irradiated group and the control groups ($p < 0.00001$), but also as compared with the group given misonidazole alone ($p < 0.01$), with the higher values for the vascular area occurring in the group given combined irradiation and misonidazole.

Figure 10 shows the percentage of vascularity in the controls, the rough data from the oedematous trachea and the figures for the treated animals when maximal correction was made. Even if the unlikely influence of a maximal 30% error in the calculation of the vascularity is considered, the significant increase of the vascularity in the groups treated with misonidazole is clear.

A correlation between the oedema and the vascular area ($p < 0.01$) was observed in the group receiving misonidazole. This correlation was also found in the group treated with misonidazole and irradiation ($p < 0.01$). No such correlation was found in the group treated with irradiation alone ($p > 0.1$).

Temperature measurements

The greatest difference in the temperature at *pars membranacea* in an individual animal in the groups treated with irradiation with and without misonidazole amounted to 1.8°C (in one animal). There was no significant relationship between dose and temperature in these experiments.

Discussion

This study determined radiation effects in two ways: effects on the oedema of the ciliary cell layer and influence on the vascularity in the subepithelial layer. When compared with the control animals, significant changes were seen in vascularity in the irradiated group. Measurements of the height of the ciliary cell layer, however, showed an oedema within the irradiated as well as the subepithelial area. This oedema was clearly dose-dependent and increased with increasing dose, indicating radiation damage to the blood vessels. Such an effect has been reported from earlier radiation research, as early as 1943 by Rigdon and Curl [20]. Their investigation found changes in the skin of the rabbit immediately following irradiation with X-rays. Injection of dye showed localization and concentration within irradiated areas, suggesting that this phenomenon resulted from changes in the permeability of the endothelial cells produced by the irradiation. Later research [19,22] found vascular permeability changes and changes in the vascular plasma volume, shown by injection of ^{125}I -labelled serum albumin. An increase in the transfer rate of plasma protein from blood vessels into extravascular space could be observed as soon as 3 h following irradiation. It is thus generally accepted that ionizing radiation has a direct effect on blood vessels, causing an increase in vascular permeability and leakage of plasma proteins into the tissue. This is a most conspicuous and easily identified phenomenon in the mucous membranes of the oral cavity during the course of fractionated radiotherapy of tumours of the head and neck. The oedema observed in the tissue is probably secondary, depending on an intensified fluid flow from the blood vessels by diffusion and osmosis.

This phenomenon is generally regarded as early radiation damage, occurring in the first hours-to-weeks after completion of irradiation and is separated from later events termed late radiation damage first occurring some months-to-years following irradiation and which is manifested as fibrosis and necrosis [7,17]. The present investigation confirms the results of earlier studies, where irradiation produced an increased height of the ciliary epithelium

of the trachea [5]. This effect was found to be dose-dependent, with an epithelial cell layer about twice as thick after a dose of 20 Gy when compared to 2.0 Gy, rapidly appearing as early as the third day after irradiation and quite long-lasting, i.e. unchanged on day 10 after the end of irradiation, when observation was stopped. When misonidazole was given in a dose roughly corresponding to the daily therapeutic doses in humans, bearing in mind the shorter half-life of the substance in rabbits, there was no measurable increased height of the ciliary epithelium.

It was most conspicuous that when misonidazole treatment was combined with irradiation, the radiation dose dependency was abolished.

It is a well-known fact that anaesthetics can affect many physiological parameters, such as the heart and the respiratory rates, the peripheral blood flow and the body temperature [15,16]. Such changes may be accompanied by primary or secondary changes in the microcirculation of the trachea. In the present study, however, where vascularity in unanaesthetized rabbits (Control a) was compared with that in the group of rabbits given anaesthetics (Control b), no difference could be detected.

The vascular area (the vascularity) in the subepithelial layer is composed mainly of capillaries. This area was increased by a factor of about 3.5 when misonidazole was given with or without irradiation. As far as we know, no published studies have reported any microcirculatory or haemodynamic effects of misonidazole. As the number of capillaries remained unchanged, the vascularity calculated in the present study must be due to an increased dilatation of the blood vessels. With morphological methods, it is not possible to determine whether this is the consequence of vascular stasis, which would lead to hypoxia, or represents an increased blood flow (hyperemia) with an improved oxygen delivery to the tissues. It has been shown, however, that tissue oxygenation following a 0.5 mg/g dose of misonidazole to anaesthetized C₃H mice increased, according to oxygen cathode readings, 2.5 times in subcutaneous tissues and 6.0 times in tumour tissues [9]. In other experiments where metronidazole,

another nitroimidazole, was given to mice, an increased tumour tissue oxygenation was registered concurrently with a decreased core temperature [14]. Since heterocyclic nitrocompounds such as the nitroimidazoles can inhibit cellular oxygen utilization and lead to an increase in radiation response in vitro [8], it may be hypothesized that the effect of misonidazole on tissue oxygen tension is possibly through a decreased overall metabolic rate. Even if the cross-sectional area of the peripheral vascular bed is only one determinant of peripheral tissue blood flow, the present results, together with other data in the literature, are compatible with the interpretation that misonidazole may also have haemodynamic effects which could increase delivery of oxygen to some normal tissues such as the trachea. It also seems reasonable to assume that an altered microcirculation with stasis or increased blood flow (hyperemia) could relate to the increased oedema observed in the ciliary epithelial layer. There is accordingly a close correlation between this factor and vascularity in the group of rabbits where misonidazole was given either alone or in combination with fractionated irradiation.

Knowledge of the behaviour of the tissue vasculature during irradiation is of great importance, not only for the understanding of cell proliferation but also for the planning of clinical radiotherapy, since the supply of oxygen, which is the most potent modifier of radiosensitivity, depends on blood circulation. The results of the present study seem to suggest that misonidazole can produce changes in microcirculation in at least one tissue of the rabbit, namely, the trachea. Such haemodynamic effects might significantly influence the therapeutic ratio in a target volume. If the trachea normally contains a significant proportion of hypoxic cells, they may be made oxic by means of increased oxygen delivery to the tissue, which should be taken into account in clinical situations.

As mentioned earlier, it cannot be ascertained with the present investigational methods whether a dilated microvasculature is secondary to stasis or is a consequence of active dilatation with increased blood flow. This seems to be an important question for further research. Hyperemia should only affect

the radiosensitivity of the trachea if this tissue is normally radiobiologically hypoxic, which does not appear to be known as yet, according to reports in the literature. Stasis, on the other hand, should reduce radiosensitivity. If a change in microcirculatory blood flow would influence the outcome of radiotherapy in the region of the therapy, this would only be due to the selectivity of this change for the normal tissue as compared with the tumour tissue.

The lack of correlation between the height of the subepithelial lamina and vascularity in the group where only irradiation was given suggests that effects of irradiation were mostly directly mediated on the endothelial cells and were not secondary to changes in the microcirculation. A correlation was found, however, when misonidazole was administered alone or in combination with irradiation, which indicates mechanisms secondary to alterations in the microcirculation.

Conclusion

Misonidazole can increase the vascular area in the subepithelial layer of the rabbit trachea. This phenomenon correlates with the development of an oedema in both the subepithelial area and in the ciliary cell layer. This was observed both when the drug was given alone and when it was combined with ionizing irradiation. Irradiation leads to no significant increase of the vascular area, but shows a dose-dependent oedema of the ciliary cell layer and the subepithelial area.

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DOSE RESPONSE STUDIES
OF SINGLE DOSE IONIZING RADIATION ON
THE CILIATED EPITHELIUM OF THE RABBIT'S TRACHEA

A physiological and ultrastructural study

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1. SUMMARY

In order to further investigate the biological effects on the ciliated epithelium of the rabbit's trachea exposed to ionizing radiation, ultrastructural and physiological examinations have been performed. Single doses of 2, 2.5, 5, 10, 15, 20, 25, 30 Gy respectively have been given and investigations made during 10 consecutive days. Low doses (2 and 2.5 Gy) cause during the first days after irradiation an increased ciliary activity which during the following days returns to normal. Ultrastructurally blebs can be observed on the cilia. Five and 10 Gy of irradiation also cause a slight increment of the ciliary activity but in that dose range the structural effects are more pronounced with swollen ciliary tips, bent tips, some cilia broken and adhering to each other. Larger single doses (15, 20, 25 and 30 Gy) cause reduced ciliary activity within the irradiated area and after 30 Gy a reduction of about 50 %. In this dose range the ciliary activity is irregular, and ultrastructurally, most cilia are broken and adhere to each other, but some of the cilia seem to be unaffected. The goblet cells respond to irradiation with an increase in number already after 2 and 2.5 Gy. Mucinous granula can be observed on the ciliary surface and after 30 Gy there is an explosive emptying of the goblet cells. The animals irradiated with 30 Gy lost in weight during the observation period. Therefore LM of oesophagus were performed. The investigations here performed show that the ciliary epithelium offers a system for the study of early radiation effects and direct comparison between physiological and ultrastructural changes.

Key Words: Trachea, Ciliary epithelium, Single dose, Ionizing radiation, Physiological activity, Scanning electron microscopy, Transmission electron microscopy, Light microscopy.

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2. INTRODUCTION

From the clinical point of view, studies of the effect of ionizing radiation on the ciliated mucous membranes of the respiratory air passages deserve special attention, since radioteraphy of malignancies in these regions is commonly connected with troublesome side-effects, originating from failure of the transport mechanism of the cilia. Thus, physiological and morphological studies have been performed on animals concerning the effect on the cilia during irradiation (4,5). The authors found mainly a stimulatory effect of the ciliary beating, but no morphological changes with doses applied to an extent of no more than 30 Gy. Examinations of ciliary reactions immediately after irradiation (hours), showed decrease and inhibition of the ciliary frequency with doses up to 70 Gy (16,25).

These investigations have been performed in vitro on a dissected rabbit's trachea. One step further to get closer to the clinical problems, was irradiation of animals in vivo. Investigations of the tracheal epithelium were performed in vitro, 1-10 days after completion of irradiation, both after fractionated irradiation (2 Gy/F) (1) and 10 Gy single dose (2). Ultrastructural changes were seen, with blebs on the cilia, and an increment of the goblet cells, connected with changes in the physiological activity of the cilia. Ten Gy single dose, caused a large number of blebs, mucinous granula, most pronounced on days 4-7, while the ciliary activity registered within the irradiation field was $\geq$ the activity within control area. The divergence in this discovery made it important to investigate the interrelation between the structural and physiological changes, as well as the influence of the dose and the period of time. Therefore in this paper experiments have been performed on the rabbit's trachea, irradiated in vivo with single doses from 2 Gy (clinical dose) to 30 Gy. Doses above 30 Gy were not considered to give any further meaningful information. As not only the trachea was affected by the irradiation but even the oesophagus with effect on the animal's capacity to take in nourishment, a special examination of the animals weight was made.

Furthermore, the temperature was measured, both in the irradiated and non-irradiated areas, to see if this was a factor to take into account in the interpretation of the results.

3. MATERIALS AND METHODS

This investigation comprises 100 rabbits, 80 of which were irradiated in groups of ten with single doses ranging from 2 Gy to 30 Gy (2, 2.5, 5, 10, 15, 20, 25 and 30 Gy). Each day during ten consecutive days, one rabbit was taken out from the group and examined, starting 24 hours after the completion of irradiation. 20 rabbits had no treatment and served as controls.

The rabbits were full-grown, weighed about 2 kg and were of different sexes and breed.

Irradiation technique: A conventional Siemens X-ray unit was used with 160 kV, HVL = 4 mm Al, SSD:50 cm, dose rate: 0.013 Gy/s. Irradiated area: 2 cm of the upper part of the trachea. The lower part of the trachea which served as a control was covered with lead. Before irradiation the animals had been rendered unconscious by intraperitoneal injection of pentobarbital (40 mg/kg bodyweight). During irradiation the animal was laid on its back with the extremities stretched out and fixed, and the absorbed dose in the trachea and oesophagus was controlled by ion-chambers and thermo-luminescens dosimeters. No dose could be measured 15 mm outside the irradiation field.

Experimental procedures: The animals were sacrificed by a blow on the skull in order to avoid pharmacological side effects. Immediately thereafter, the temperature was measured with a thermistor beneath the pars membranacea within the irradiated area and also within the area which served as a control. Then the trachea was immediately dissected out in its entire length (7 cm) and samples were taken (and fixed) from the irradiated area in the upper part of the trachea (T1) and from the control area in the lower part of the trachea (T2) adjacent to the tracheal bifurcation. The trachea was divided into two halves which were placed in a chamber with a controlled temperature (37 °C) and humidity (90 %). Through an opening in pars membranacea the ciliary activity could be registred by a method developed and described by Håkansson and Toremalm (18).

3.1. Preparation for SEM

The specimens were transferred into 2.5 % glutaraldehyde in 0.15 M cacodylate buffer (pH = 7.3), for 12 hours. They were then transferred into the same buffer and later osmium fixed in 1 % osmium tetroxide in 0.15 M cacodylate-buffer for two hours.

Dehydration took place with graded series of ethanol and then the specimens were transferred to Freon TF 618. Later on the specimens were dried according to the critical point method in a Balzer 000 Critical Point Dryer. They were finally sputter-coated with gold plus palladium in a Polaron SEM Coating unit E 5000. Then they were examined in a Cambridge Stereoscan MARK IIA, or a Ziess Nanolab Electron Microscope. The microscopes were operated at 20 kV.

3.2. Preparation for TEM

The samples were fixed as for the SEM - preparations and also in 1 % OsO₄ in phosphate buffer (pH 7.4) for two hours, rinsed in 0.15 M cacodylate buffer, dehydrated in alcohol and embedded in Vestopal W or Epon. Sections of 1 μ m thickness were cut out on an LKB - Ultratome, stained with toluidine blue and examined in a light microscope. Ultrathin sections were cut out and contrasted with lead citrate and uranyl acetate, or en bloc with 0.5 % uranyl acetate. A Ziess EM 10 Electron Microscope was used.

3.3. Preparation for LM

Sections for LM were fixed and embedded as for TEM sectioning. 1 μ m thick sections were cut with an LKB-Ultratome and stained in methyleneblue and examined in a Zeiss light microscope.

All the preparations were examined with LM and SEM and about 30 % of the preparations with TEM (Fig. 1).

3.4. Statistical analyses

Variance analysis (BMPD) was used for the evaluation of changes in ciliary activity.

3.5. Determination of body-weight

The weight of the animal was checked before the treatment and then daily after the treatment had been given.

4. RESULTS

Fig. 1 shows the irradiation schedule for the different single doses. From each tracheal specimen registration of the physiological activity from the upper part (T1) and the lower part (T2) was made. Morphological investigations were also performed with LM, SEM and TEM.

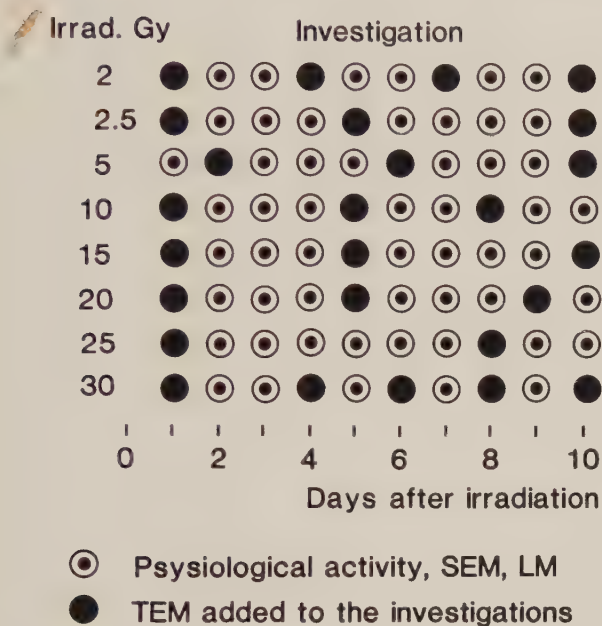


Fig. 1: Irradiation schedule showing the different single doses and days when investigations were performed.

4.1. Physiological activity (ciliary activity)

In order to determine whether the irradiation of the upper part of the trachea (T1) influenced the ciliary activity of the lower part of the trachea (T2), two different types of control measurements were performed. This is illustrated in Table 1.

TABLE 1. Schedule showing ciliary activity in the upper part of the trachea (T1) and the lower part (T2) for the 20 control animals, and the 10 animals in each radiation group respectively. Each value represents the mean value for the group $\pm$ SD.

Untreated animals		Dose (Gy)	Treated animals	
(Control a.)			Irradiated part	Non irradi. part (Control b.)
T ₁	T ₂			
1096 ± 36	1104 ± 33	2	1216 ± 44	1190 ± 31
		2.5	1162 ± 41	1125 ± 41
		5	1245 ± 30	1212 ± 49
		10	1270 ± 73	1188 ± 37
		15	1152 ± 66	1254 ± 36
		20	1018 ± 64	1266 ± 41
		25	957 ± 72	1252 ± 35
		30	660 ± 101	1192 ± 55

Control a: Recording of the ciliary activity in the upper and lower part of the trachea in 20 untreated animals. In these measurements, the ciliary activity was about 1100 waves/min (Table 1), T₁, T₂ and the quotient (T₁/T₂) was found to be 1. The same results was obtained whether registration started from the upper or lower part of the trachea and the frequency did not change during the 30 minutes of registration. This agrees with the result from Fujiwara et al (15) where he reported intact ciliary activity during 3-4 hours in vitro in a chamber with controlled air and humidity.

Control b: Control measurements from the lower part (T₂) of the irradiated animals showed a ciliary activity which was about the same as in the control animals (Table 1). The terminology normally used 'ciliary beat frequency' (1,2) is in this paper changed to 'ciliary activity' and 'beats/min' to 'waves/min'. The reason for this change in terminology is the pronounced ultrastructural damage which were observed after the high single doses >10 Gy. Also in the microscope it could be observed how the carpet of mucus stood still in certain places, whereas only isolated reflexes could be seen. This damage, was considered to cause lower efficiency in the stroke of the cilia and reduced transport capacity and was observed when Indian ink was placed on the ciliary carpet. With single doses of 10 Gy and higher, the ink moved around in circles. The amplitude of the registered oscillations

TABLE 2. Schedule showing the ciliary activity quotient (T1/T2) for each rabbit in each group during the ten days of observation.

Dose Gy	Days after end of irradiation									
	1	2	3	4	5	6	7	8	9	10
2	1.12	1.18	0.93	0.98	1.10	1.03	0.94	0.99	1.00	0.99
2.5	1.19	1.10	1.00	1.00	1.00	0.98	1.00	1.02	0.98	1.00
5	1.05	1.00	1.09	1.00	0.98	0.97	1.06	1.05	1.01	1.01
10	1.12	1.03	1.12	1.08	1.18	1.02	1.09	1.00	1.00	1.04
15	0.96	0.88	0.91	0.92	0.92	0.87	0.88	0.97	0.83	0.94
20	0.86	0.82	0.87	0.84	0.82	0.82	0.73	0.76	0.79	0.72
25	0.79	0.79	0.77	0.78	0.74	0.81	0.78	0.65	0.77	0.72
30	0.58	0.69	0.64	0.55	0.49	0.53	0.54	0.48	0.45	0.59

was lower as compared to those of the control group and the ciliary activity was irregular which was reflected in higher standard deviation with an increasing dose (Table 1). Table 1 shows mean values for all 10 animals in each group, both from irradiated and non-irradiated areas, and Table 2 shows the quotient (T1/T2) each day after irradiation for each group.

Irradiation with 2 and 2.5 Gy caused an increase in ciliary activity (about 10-20 %; Table 2) the first days after the completion of the irradiation, which during the following days returned to normal. During the whole period, for both groups, the ciliary activity within the irradiated areas was slightly above normal (about 2 %; Table 1, Fig. 2). Each point in Fig. 2 represents the mean value of the quotients from the investigations during day 1 to 10 after irradiation. The 5 Gy single dose also caused an increased ciliary activity in the irradiated part of the trachea, most clearly shown on day 3 (9 %; Table 2), and for the whole period the ciliary activity in the irradiated part was slightly increased (2 %; Table 1, Fig. 2).

After 10 Gy the ciliary activity in this series is at its highest on day 5: 18 % (Table 2) and for the whole observation period it was 6 % (Fig. 2). With this dose irregular ciliary activity is first observed. With an increasing dose the ciliary activity within the irradiated areas decreased (Fig.2). After 15 Gy the activity was reduced to about 10 %, after 20 Gy to about 20 %, after 25 Gy to about 25 % and after 30 Gy the ciliary activity was found to be 50 % of its

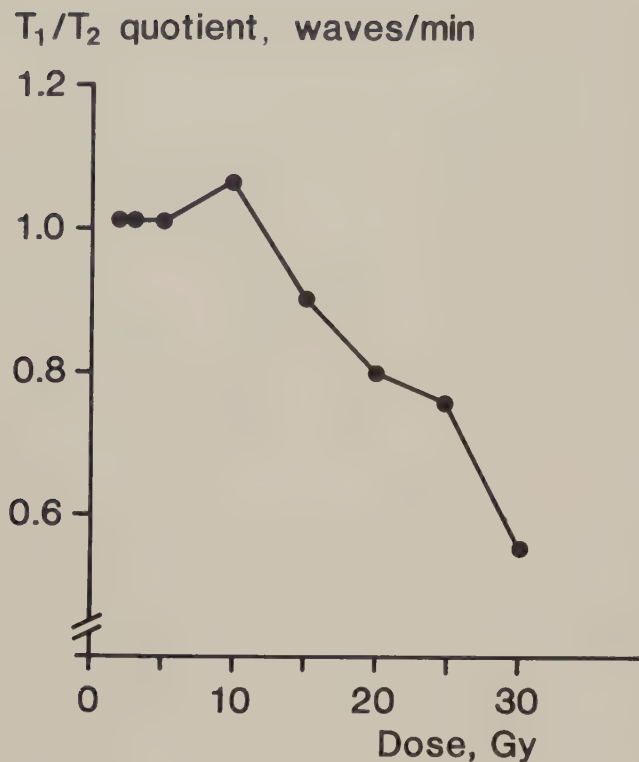


Fig. 2: The beat frequency quotient T_1/T_2 in relation to the dose given. Each point represents the mean value from all ten animals in each group.

normal value (Table 1, Fig. 2). The reduction was obtained already during the first days of observation and remained during all the days of investigation (Table 1). Statistical analysis of the material showed a significant dose effect, both linear and quadratic with reduced ciliary activity within the irradiated area and with an increasing dose ($p < 0.0001$), and a tendency to reduced ciliary activity with increasing time.

4.2. Ultrastructure

SEM of the normal tracheal epithelium showed long gracile cilia (about 7 μm in length), arranged regularly in numbers of about 200 on each cell. A few blebs were observed on the side of a cilium. In the surface epithelium goblet cells were interspaced. These had microvilli with a length of 1 μm and could be seen protruding on the surface before emptying their mucus (Fig. 3 a,b).

Radiation caused changes both on the cilia and the goblet cells (described separately).



Fig. 3 a: SEM-picture of normal tracheal epithelium. Note the gracile, regularly arranged cilia and goblet cells with microvilli (arrows).

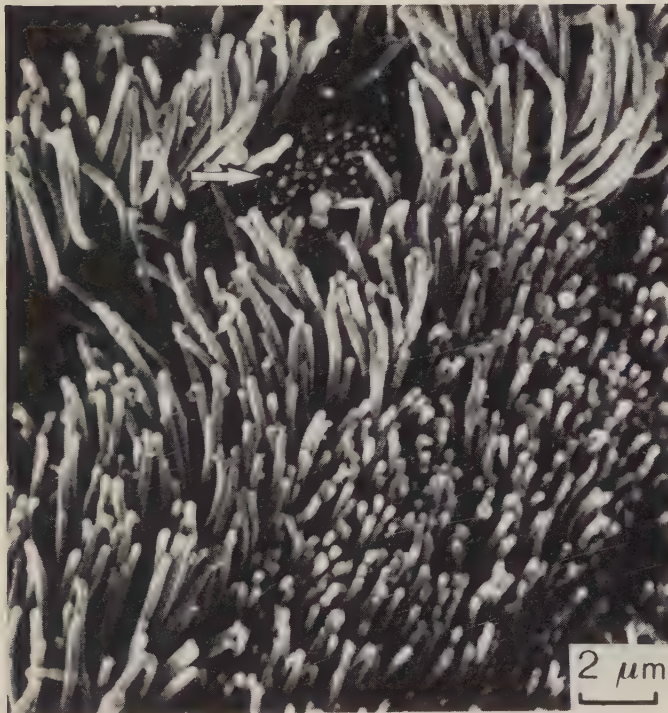


Fig. 3 b: Higher magnification of 3 a.

4.3. Radiation effects on the cilia

With increasing dose a gradual development of damage could be observed on the cilia. The first effect observed was an increment of blebs on the side of the cilia (Fig. 4 a,b). Thereafter the tips became swollen, often blown up like balloons (Fig 5 a,b) bent to a curved shape (Fig 6) and finally the cilia became short, stubbly, broken and adhered to each other (Fig 7 a,b,c, 8a,b). The changes: blebs, swollen tips, curves, broken tips and adhesion were scored from the SEM-pictures and graded on a scale from 0-2, where 0=normal ultrastructure, 1=more than normal, 2=much more than normal. Each picture was judged by 3 persons and for each dose group 10 pictures were scored, so maximally 60 points could be obtained in each group. The results from these judgements are presented in figures 9 and 10. The phenomena mentioned above are described in 4.3.1. - 4.3.3.

4.3.1. Blebs

Blebs on the cilia are vesicles on the side of the cilium (Fig. 4 a,b). TEM pictures showed that the blebs are membrane- equipped protuberances budding of the cilium. The size and number of blebs on the cilium varied, and they were occasionally observed in the control group, but the number was increased after irradiation. The greatest number was observed after 2 and 2.5 Gy. Blebs were also observed in increasing numbers after 5 and 10 Gy (Fig.9). At single doses of 15 Gy and higher, these phenomenon could no longer be observed, in a higher frequency than in the control group.

4.3.2. Swollen and bent ciliary tips

Swollen and bent tips is the next step in the damage process to the cilium (Fig 5 a,b and 6). The tips of the cilia became swollen, like balloons. A few swollen tips could be observed already after 2 Gy, more pronounced after 5 Gy and throughout the series (Fig. 9). The

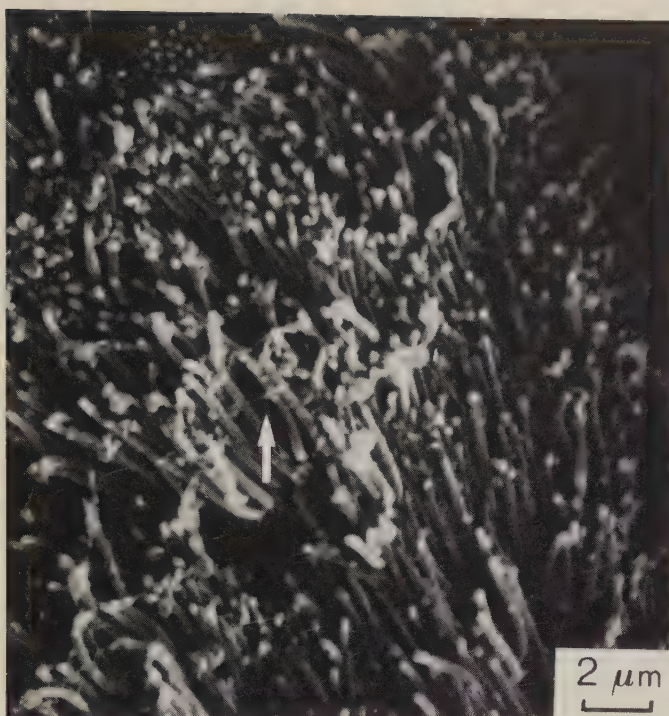


Fig. 4 a: Irradiation 2 Gy. SEM-picture. An increased amount of blebs on the cilium (arrow).

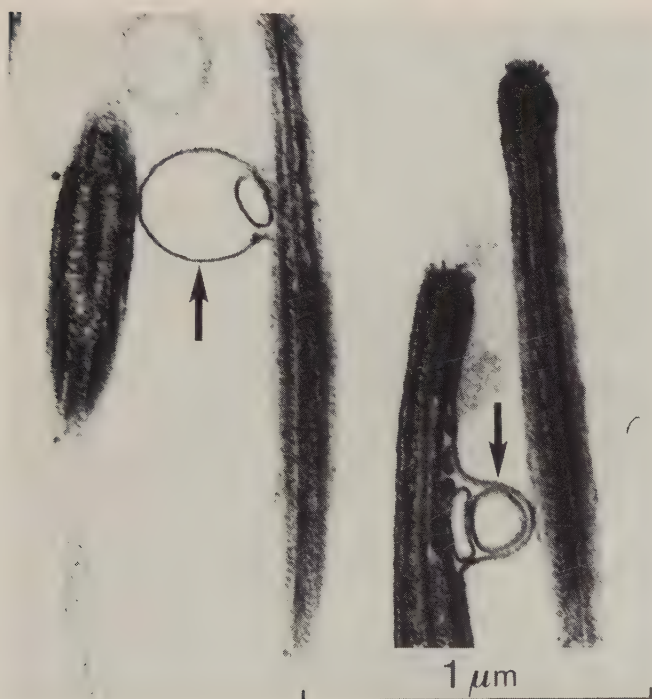


Fig. 4 b: TEM-picture illustrating the blebs on the side of the cilium (arrows).



Fig. 5 a: Irradiation 2.5 Gy. SEM-picture. Swollen ciliary tips (arrows).

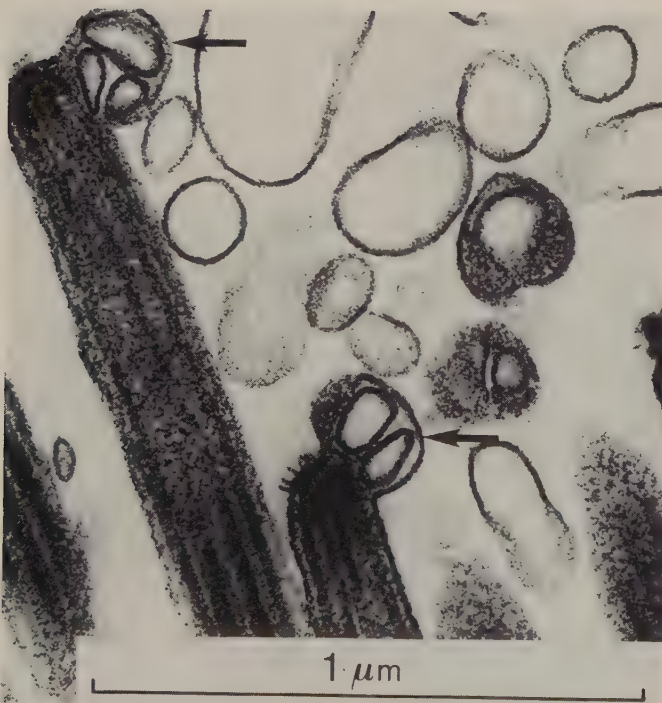


Fig. 5 b: TEM-picture illustrating the blown-up, swollen ciliary tips (arrows).



Fig. 6: Irradiation 10 Gy. SEM-picture. Bent ciliary tips (arrows).



Fig. 7 a: Irradiation 20 Gy. SEM-picture. Broken ciliary tips (arrows).

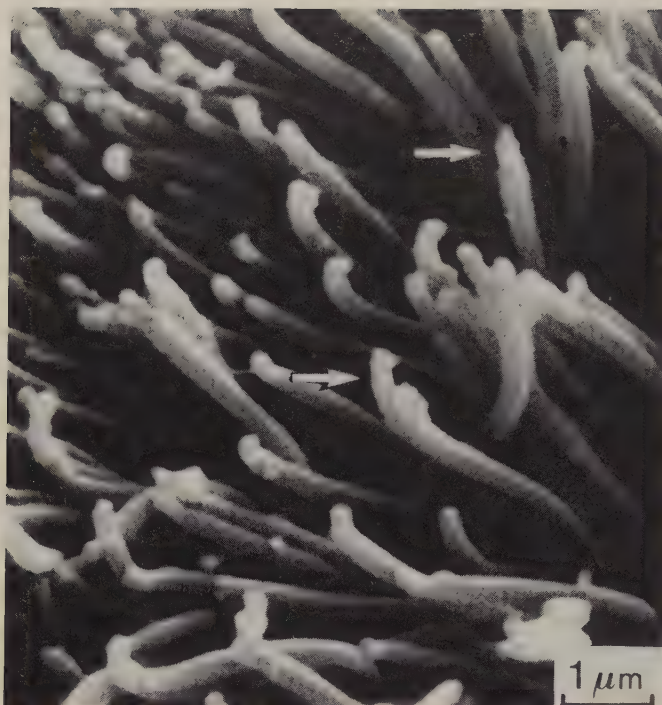


Fig. 7 b: Higher magnification of 7 a (broken tips indicated by arrows).



Fig. 7c: TEM-picture illustrating a damaged ciliary tip (arrow).

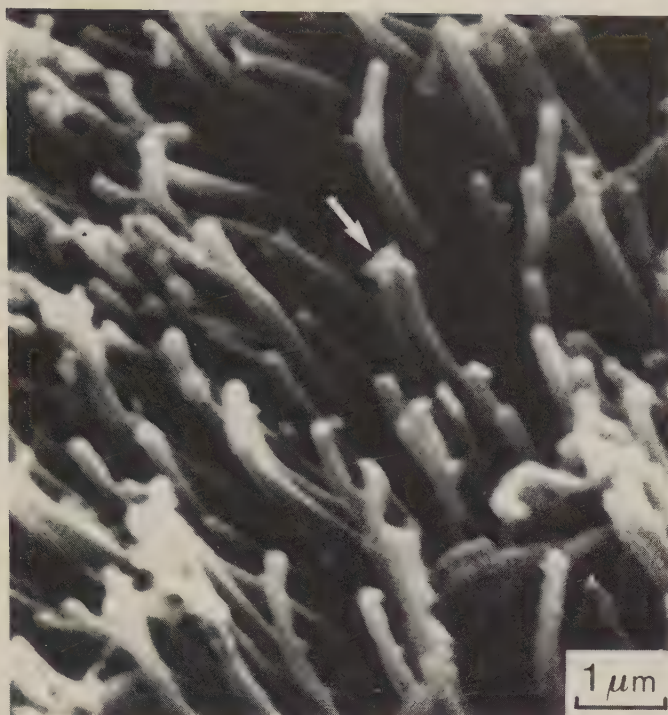


Fig. 8 a: Irradiation 30 Gy. SEM-picture. Cilia adhering to each other (arrow).



Fig. 8 b: SEM-picture. A fibrillar network connecting groups of cilia.

Average occurrence

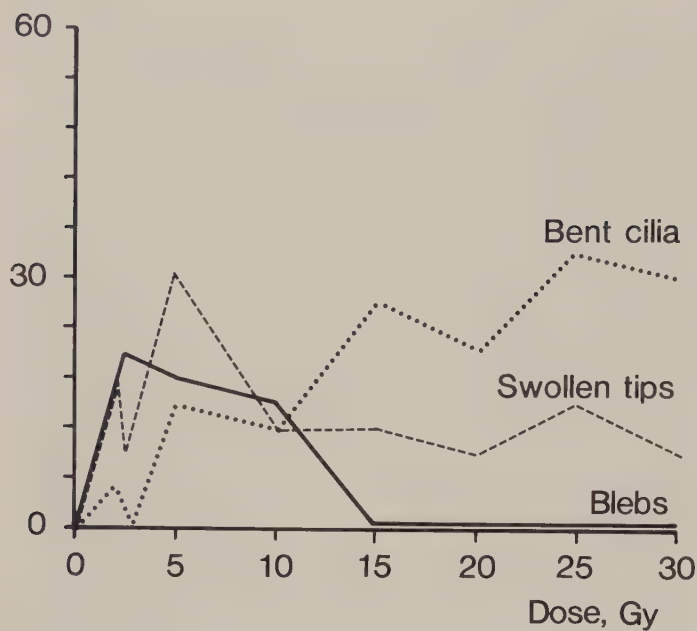


Fig. 9: Score in arbitrary units on a gliding scale expressed as mean value for each dose during ten days after irradiation as judged by three persons. 0=normal, 60=greatest abnormality.

Average occurrence

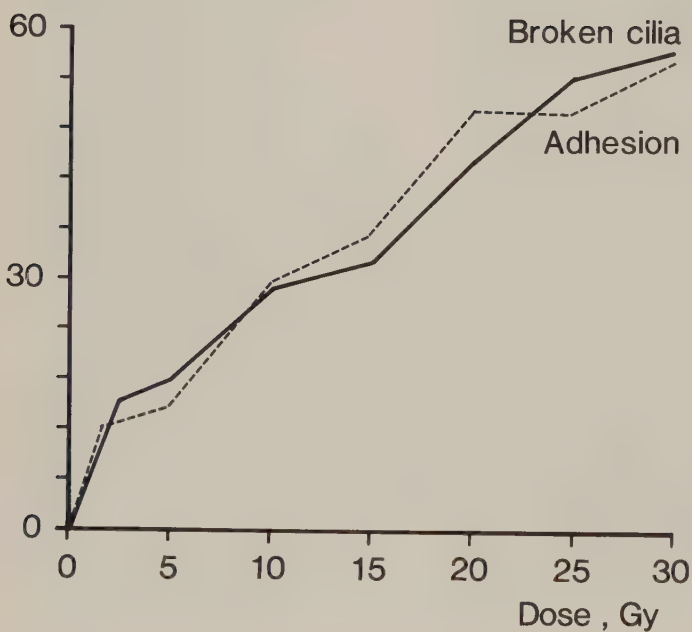


Fig. 10: Score in arbitrary units on a gliding scale expressed as mean value for each dose during ten days after irradiation as judged by three persons. 0=normal, 60=greatest abnormality.

tips got thinner and were bent. It appeared as if the blebs were budding off followed by an extraciliary withdrawal of cytoplasm before the next phase of damage started.

4.3.3. Broken cilia and adhesion of their tips

Broken cilia and adhesion (Fig. 7a,b,c, 8a,b) seemed to be two connected phenomena expressing an increased damage. These two phenomena appeared often simultaneously and were dose related (Fig. 10). Already after 2 Gy isolated broken and adhered cilia could be observed but after 30 Gy the phenomena were usually occurring and then on the whole the surface of the cilia had taken on a short and stubby appearance. Cilia were joined by a fine fibrillar network. After 15 Gy single dose and larger, the number of cilia per ciliary cell on some of the surfaces seemed to be decreased (Fig. 11 a) but the surface was covered with microvilli. Some of the remaining cilia observed apparently had an intact ciliary crown (Fig 11 b). The effects described above can be found without any clear variation during all the 10 observation days, which is why the appearance of the changes for each dose group are gathered at one point.

4.4. Goblet cells

Goblet cells are a vital component in the respiratory epithelium. From these cells a mucous secretion is produced, which lies like a protective carpet over the cilia and in which the transportation of particles occurs. SEM of the tracheal-epithelium shows widespread goblet cells in the epithelium (Fig. 3 a,b). The surface of the mucous cell is covered with microvilli, 1 μm in length.

After 2 Gy single dose, an increased number of goblet cells can be observed in the epithelium (Fig. 12 a,b). These have a normal structure. After 2.5 Gy, an increased number of goblet cells can be observed, but also mucinous granula on the surface of the cilia. At 10 Gy a large number of mucinous granula can be seen (Fig. 13) and after

30 Gy, an explosive emptying of the goblet cells over the surface initially takes place (Fig. 14 a,b). Fig. 15 is an estimation of the number of goblet cells and mucinous granula during the whole observation period for each single dose.

4.5. Effect on weight and oesophagus

All the experimental animals were weighed before the commencement of the treatment and after this daily. Ten Gy single dose and lower induced a stable or increasing weight during the observation period (Fig. 16): 15 and 20 Gy induced a decrease in the body weight by about 5 % in the first days following the treatment, but on day 10 the animal had regained its original weight. LM of oesophagus showed after 30 Gy, single dose, within the irradiated region edema in the mucosal layer, inflammatory infiltrate in the submucosa and a thinned, damaged surface epithelium.

4.6. Temperature measurements

With a thermistor, temperature measurements were made from the irradiated part beneath pars membranacea and compared with value from the non-irradiated part of pars membranacea in the same animal. No significant temperature increase with an increasing dose was found. The registred temperature was about 38° C.

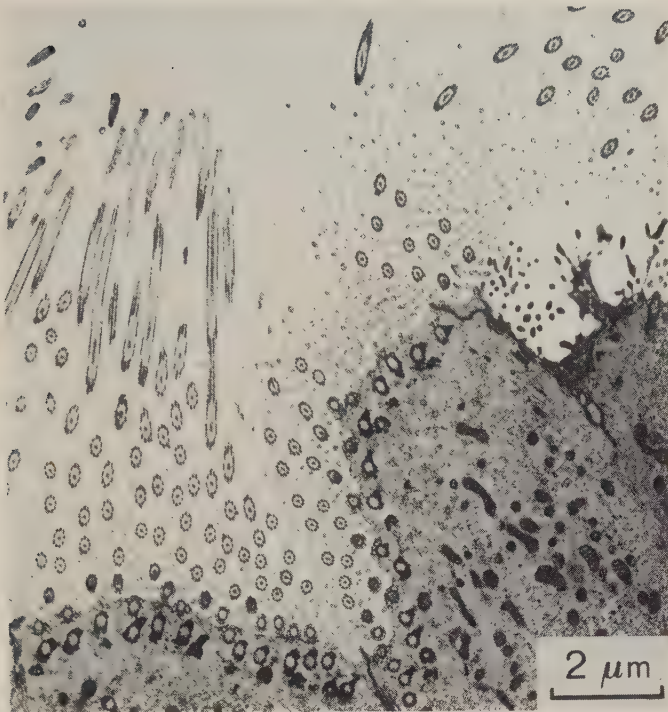


Fig. 11 a: TEM-picture with an appearance of a reduced number of cilia on the surface of one ciliated cell.

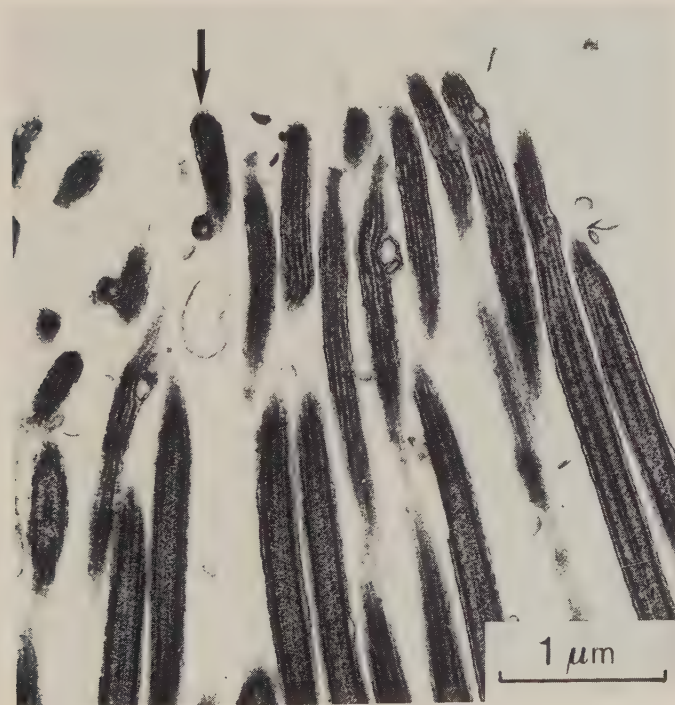


Fig. 11 b: Higher magnification of the cilia in 11 a. Apparently intact ciliary crown (arrow).



Fig. 12 a: Irradiated ciliary epithelium 2 Gy. SEM-picture. Note the increased amount of goblet cells (arrows).



Fig 12 b: Higher magnification of 12 a.

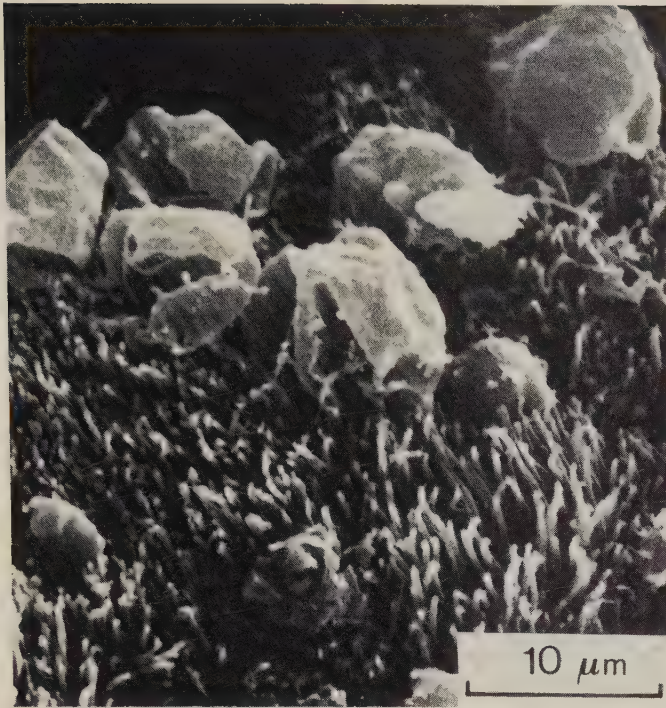


Fig. 13: 10 Gy
irradiation. SEM-picture.
Mucinous granula on the
ciliary surface.



Fig. 14 a: 30 Gy irradiation. SEM-picture. The whole ciliary surface covered with mucinous granula.

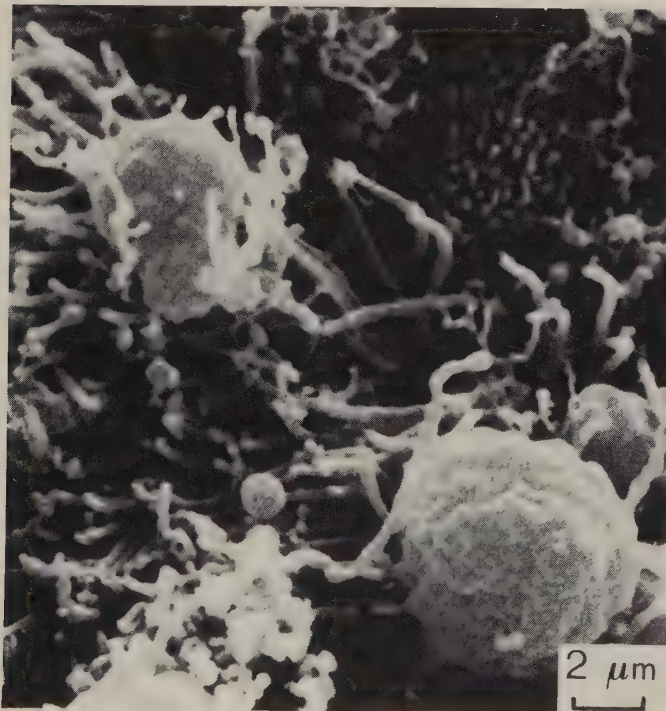


Fig. 14 b: Higher magnification of 14 a.

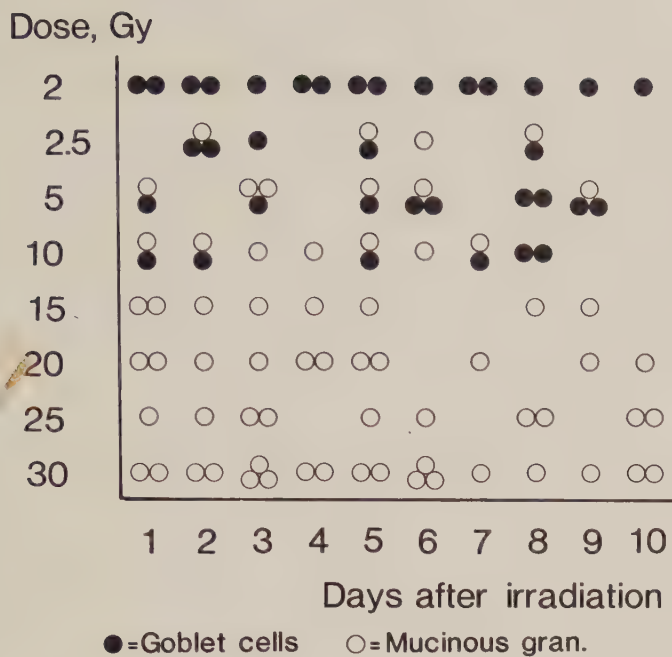


Fig. 15: Estimation from SEM-pictures of the number of goblet cells and mucinous granula on the ciliary surface:

● = more goblet cells than normal
 ●● = many more goblet cells than normal
 ○ = single mucinous granula
 ○○ = many mucinous granula
 ○○○ = the surface covered by a large number of mucinous granula

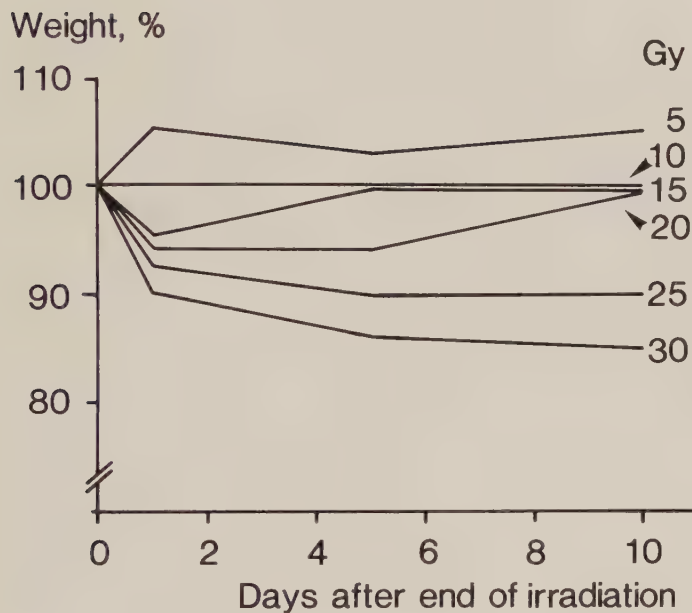


Fig. 16: Weight curve. Percent change in weight after irradiation with single doses of 2-30 Gy.

5. DISCUSSION

The lung and the trachea offer a contrast in irradiation response where the adult lung is a tissue with slow cellular proliferation (26) while the trachea is a rapid cell renewal system. LD 50 for the mouse lung is 1300 rad and death occurs between 80 and 180 days (11). Measurements of breathing rate in mice after irradiation enabled an acute phase of pneumonitis (2 - 5 months) and a late phase of fibrosis (after 8 months) to be distinguished (13). Thus late effects (months) of irradiation is found in the lung and early effects (days-weeks) is expected in the trachea. Investigations of the irradiation effects on the ciliated cells have been taking place for very many years e.g. since 1903, when Bohn found a stimulation of ciliary activity among sea-urchin eggs when they were subjected to irradiation and in 1939, Frenckner reported the effect of X-ray and Radium upon the action of cilia within the respiratory tract (nasal mucous membrane) where an arrest of the movement of the cilia was described (14). Ogi (25) and Fujiwara (16) found a decreased ciliary activity in the tracheal epithelium after completion of irradiation, while Baldetorp (4,5) found an increased ciliary activity in the tracheal epithelium during irradiation. All these investigations were performed in vitro and no structural effects could be observed either with LM, SEM or TEM. Irradiation of tracheal epithelium in vivo with fractionated irradiation and 10 Gy single dose (1,2) where physiological and ultrastructural measurements were performed (1-10 days after completion of irradiation) showed dose-dependent effects on the parameters observed. To bring more clarity to the mechanisms with earlier irradiation effects, it was considered important to carry out further experiments with single doses. Regarding ultrastructural changes these are crystallized, in the experiments performed here, in a pattern of irradiation effects on the cilia, where blebs on the side of the cilium seems to be an early phenomenon, noticable already after 2 Gy. It is believed to be an early sign of radiation induced membrane damage, although the blebs are normally observed in the untreated trachea to a lower extent (7). Perhaps it represents weak regions on the cilia, that are more easily affected.

The fact that ionizing radiation causes cellular and subcellular membrane damages has been observed in a number of investigations (13, 17, 18, 31, 32). The next stage in the development of ciliary damage seems to be swollen ciliary tips and bent tips (Fig. 5, 6). Cytoplasm are loosened extraciliary and the cilia adhere to one another (Fig. 7, 8). This is thought to illustrate a graded system in the effects of damage where the damage is observed in different degrees with different doses. A hypothesis is that the blebs are the earliest sign of damage that is possible to observe and which can be seen already after 2 Gy. Blebs are not seen with large single doses, 15-30 Gy (Fig. 9), in greater numbers than in the control material. With 30 Gy broken tips and adhesion usually appear in great numbers (Fig. 9, 10) which can be interpreted as that broken tips and adhesion is the greatest damage that can be observed here. With doses of 20 Gy or larger, the number of cilia in each cell is thought to be reduced (Fig 11 a), in spite of the fact that the average number of ciliary roots remained within the membrane. This may depend on the fact that some cilia have broken off at the base. Intact cilia can be observed and even cilia with intact ciliary crowns (Fig. 11 b). The fibrillar network connecting different groups of cilia, is supposed to have protein components, caused by protein leakage from the ciliary epithelium since the normal mucus consists of mucopolysaccharides which are not fixed with the conventional methods here used. These ultrastructural changes are observed in this tissue already the first day after irradiation and are maintained during the ten days of observation. No obvious signs of repair can be observed during this period. The existence of repair of irradiation induced damage in the tracheal tissue is quite plausible, but cannot convincingly be defined with the methods here used.

Different kinds of repair mechanisms has been known since 1959 when Elkind and Sutton first described the repair of sublethal radiation damage in cell cultures taking place minutes to hours after irradiation (9). The rate of sublethal damage depends on the size of the dose. This has been observed in the spinal cord of rats with faster repair after smaller fractions (3). Slow repair has been

described for lungs (11, 13) with a time scale of hours to days observed both histologically and by measurement of breathing rate.

During the 10 days of observation no signs of repopulation were seen in any of the doses and probably the time was too short for this event to take place. The turn-over rate for ciliary epithelium is about 2 weeks. Compensatory proliferation does not occur until cells have died and the resulting cell depletion has been recognized, which in gut occurs within 2-3 days (33), in skin and buccal mucosa it begins within 1-2 weeks (8).

The effects of irradiation were also observed on the goblet cells. At low dose range (2, 2.5 Gy) an estimation of the number of goblet cells and mucinous granula was made in relation to the normal tracheal epithelium. After 2.5 Gy solitary mucinous granula on the ciliary carpet were observed. The granula increased in number with larger doses, and after 30 Gy an explosive extrusion of mucinous granula was seen on day 1 after irradiation and an increased amount of mucus was thereafter observed during all 10 days (Fig. 14 a, b). The stimulatory effect of irradiation on goblet cells has been observed earlier, both in the ciliary epithelium (1) and in the intestine (15). The mechanisms behind this are not clear. The ciliary epithelium consists of basal cells, intermediate cells, apical ciliated cells and goblet cells. It is not known if goblet cells are produced from undifferentiated basal cells or are differentiated from intermediate cells. The increased number may result from increased mitotic activity or increased turnover rate of the ciliary epithelium with increased production of goblet cells. The great amount of mucus plays a major role in the ciliary activity registered (28) and is probably an important factor in the reduction of ciliary activity, among other things as a result of changed viscosity of mucus.

Ciliary activity can further be divided into three phases, where in the low dose range (2, 2.5 Gy) an increase in the ciliary activity of about 10 % is observed during the first days of observation which is interpreted as related to an increased amount of mitochondria

apically in the cells and an increased amount of ATP available (1). Within this dose range the ciliary carpet is regular and, the recorded oscillations are regular. Ten Gy also causes increased ciliary activity during the observation period (Fig. 2), here, however, the oscillations observed are low and irregular. Indian Ink, when placed on the ciliary carpet moves around in circles and ultrastructurally, damage on the cilia can be seen and an increased amount of mucus (2). These observations indicate that even if the ciliary activity is increased within the irradiated area, the effectiveness of the ciliary carpet to move is reduced, perhaps also through the influence on the ciliary crown. Further increment of the dose caused reduction of ciliary activity and after 30 Gy the activity within the irradiated part was reduced by about 50 %. Here, ultrastructurally, the number of cilia on each cell was reduced, adhesion and broken cilia were frequently observed so the cilia that were able to perform any activity were damaged.

The laboratory animals are of different breeds and sex and ciliary activity varies from one animal to another. Therefore the irradiated area for each animal is compared to the control area in the same animal. In Table 1, however, the different animals from each group are compared. Control a (untreated animal) has a ciliary activity of about 1100 waves/minute and in control b (lower part of the trachea T2, in an animal which is irradiated in the upper part T1) ciliary activity varies between 1096-1254 waves/min. These differences between the control animals are small, and not correlated to the dose given, nor are any structural changes observed in the control part with LM, SEM and TEM and it is not believed that any diffusion flare (10, 22) exists in these investigations.

6. CONCLUSION

Radiation causes physiological and ultrastructural effects on the ciliated epithelium of the rabbit's trachea, observable during the first ten days after irradiation. Physiologically after 2 and 2.5 Gy increased ciliary activity (about 10 %) is observed the first days after irradiation, after which it returns to normal. After 10 Gy ciliary activity is also heightened but the ciliary carpet as a whole has a reduced ciliary activity. Larger doses: 15, 20, 25 and 30 Gy cause reduced ciliary activity, after 30 Gy by about 50 %. Ultrastructurally, blebs are observed on the cilia after 2 Gy. With larger doses the tips became swollen, bent and broken, adhering to each other and reduced in number. No signs of repair or repopulation were observed during the observation period.

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DETACHED CILIARY CELLS

